MEDICAL LABORATORY TECHNIQUES
FOR ROUTINE DIAGNOSTIC TESTS

Volume VII

CLINICAL BIOCHEMISTRY

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Project Coordinator

राष्ट्रीय शैक्षिक अनुसंधान और प्रशिक्षण परिषद्
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FOREWORD

The programme of vocationalization of higher secondary education has been accepted by the country as it holds forth great promise for linking education with the productivity and economic development of the country by providing education for better employability of the youth

In view of the importance of the programme the NCERT is making an all out effort to provide academic support to the implementing agencies in the States One of the major contributions of NCERT is in the field of curriculum development and in the development of model instructional materials. The materials are developed through workshops in which experts, subject specialists, employers' representatives, curriculum framers and teachers of the vocational course are involved

The present volume on **Clinical Biochemistry** is one of the series on "Medical Laboratory Techniques for routine diagnostic tests" and is meant for the students of Medical Laboratory Technician course It is being published for wider dissemination amongst students and teachers throughout the country. I hope that they will find the volume useful.

I am grateful to those who have contributed to the development of this volume I must acknowledge also the immense interest taken by Prof. A K. Mishra, Head, Department of Vocationalization of Education in inspiring his colleagues in their endeavours to develop instructional materials. Shri G Guru, Reader, functioned as the Project Coordinator for the development of this title He has my appreciation and thanks for planning, designing and conducting the workshop, for technical editing and for seeing this title through the press

Suggestions for improvement of this volume will be welcome.

P.L. MALHOTRA
Director
National Council of Educational
Research & Training

New Delhi,
March, 1986

PREFACE

Ever since the introduction of vocationalization in our school system by several States and Union Territories in our country the paucity of appropriate instructional materials has been felt as one of the major constraints in implementation of the programme and a source of great hardship to pupils opting for vocational studies at the higher secondary stage

The Department of Vocationalization of Education of the National Council of Educational Research & Training, New Delhi has started a modest programme of developing instructional materials of diverse types to fill up this void in all major areas of vocational education The task is too gignatic to be completed by any single agency but the model materials being developed by us might provide guidance and impetus to the authors and agencies desiring to contribute in this area. These are based on the national guidelines developed by a working group of experts constituted by NCERT

The present volume is on **Clinical Biochemistry** and is meant for the pupils and the teachers of Medical Laboratory Technician vocation being offered in a number of States and Union Territories. It contains laboratory procedures and test details of routine biochemical tests required to be performed by a Laboratory technician. It is hoped that the users will find it immensely useful

The material was developed during the workshop held in Dr T.M A Pai Research Centre, Manipal The present version was finalised after incorporating suggestions and comments by experts. The names of the contributors/reviewers are mentioned elsewhere and their contributions are admirably acknowledged Shri G Guru, Reader and Coordinator of this Project, Department of Vocationalization of Education, deserves special thanks for bringing the manual in the present form The assistance of all in the Dr. T.M.A. Research Centre Manipal, especially of Dr A Krishna Rao,

Dean, Kasturba Medical College, Manipal and that of the Department of Vocationalization of Education, are also thankfully acknowledged

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March, 1986.

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The Participation of the following experts in the development of the present title as contributors/reviewers is gratefully acknowledged

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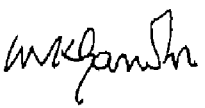
GANDHIJI'S TALISMAN

"I will give you a talisman Whenever you are in doubt or when the self becomes too much with you, apply the following test :

Recall the face of the poorest and the weakest man whom you may have seen and ask yourself if the step you contemplate is going to be of any use to him. Will he gain anything by it ? Will it restore him to a control over his own life and destiny ? In other words, will it lead to Swaraj for the hungry and spiritually starving millions ?

Then you will find your doubts and your self melting away."



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PART I

Introduction

1.1 Scope of Clinical Biochemistry

Clinical Biochemistry deals with biochemical aspects involved in various clinical disorders. In the practical clinical biochemistry, various chemical tests, both qualitative and quantitative, that are used in the diagnosis and the assessment of prognosis and treatment of the disease are dealt

The chemical tests are performed on various biological fluids such as blood, plasma, serum, urine, cerebrospinal fluid, ascitic fluid and pleural fluid or tissues (biopsy specimens). For example, when blood glucose level is higher than normal and glucose is also present in urine, the common disease entity is diabetes mellitus.

1.2 Common Tests Carried Out in Clinical Biochemistry Lab.

Although is it difficult to be familiar with all the clinical chemical tests, some of the tests that are commonly used are to be known to the technical staff involved The common tests are listed in the following table and the test details are given in page number mentioned.

2 Table VII-I Common Biochemical Tests in Clinical Practice

Sample	*Test*	*Test type*	*Test detail-page number*
URINE	Specific gravity	Urinometer	78
	Albumin	Quantitative	94
	Glucose (sugar)	Semiquantitative	93
		Quantative	95
	Blood	Qualitative	93
	Ketone bodies	Qualitative	93
	Bile salts	Qualitative	93
	Bile pigment	Qualitative	93
	Urobilinogen	Qualitative	82
	Urea	Qualitative	82
		Quantative	85
	Creatinine	Qualitative	82
		Quantative	87
	Uric acid	Qualitative	84
		Quantative	86 (119)
	Phosphate	Qualitative	83
		Quantative	87 (138)
	Chloride	Qualitative	83
		Quantative	86 (133)
	Sodium	Quantative	87 (129)
	Potassium	Quantative	87 (129)
	Urea clearance test	Quantative	89
	Creatinine clearance test	Quantative	89
	Test for lactosuria	Qualitative	98

	Test for pentosuria	Qualitative	98
	Test for fructosuria	Qualitative	98
	Test for alkaptonuria	Qualitative	98
	Test for phenylketonuria	Qualitative	98
	Titratable acidity	Quantative	87
	Ammonia	Quantative	87
GASTRIC JUICE	Hydrochloric acid	Qualitative	168
	Pepsin	Qualitative	169
	Bile salts	Qualitative	169
	Blood	Qualitative	169
	Starch	Qualitative	169
	Free acidity	Quantative	169
	Total acidity	Quantative	169
BLOOD	Blood sugar (Blood Glucose)	Quantative	103, 106
	Blood urea	Quantative	112, 114
	Serum creatinine	Quantative	117
	Serum uric acid	Quantative	111
	Serum total protein	Quantative	122
	Serum albumin	Quantative	122, 126
	Serum globulin	Quantative	122, 126
	Serum protein electrophoresis	Semi quantitative	69
	Zinc turbidity in serum	Quantitative	127
	Serum sodium	Quantative	129
	Serum potassium	Quantative	129
	Serum chloride	Quantative	133
	Serum calcium	Quantative	134
	Serum phosphate	Quantative	138
	Serum GOT (AST)	Quantative	142
	Serum GPT (ALT)	Quantative	144
	Serum alkaline phosphatase	Quantative	147
	Serum acid phosphatase	Quantative	153
	Serum amylase	Quantative	154
	Serum total bilirubin	Quantative	157

	Serum direct bilirubin	Quantative	158
	Serum cholesterol	Quantative	160
CEREBRO-SPINAL FLUID/ ASCITIC PLEURAL FLUID	Total protein	Quantative	177
	Sugar	Quantative	175
	Chloride	Quantative	179
	CSF pandy's test	Quantative	181

PART II Basic Laboratory Requirements

2.1 Glasswares

Selection of right glasswares and their purity or cleanliness is important This chapter deals with some common glasswares (Fig. VII—1, A&B)

2.1.1 Test Tube

It is a narrow glass tube closed at one end and open at the other. While using, hold it between the thumb and the index finger in a slanting position. While boiling any solution hold it with a test tube holder. While adding any liquid it should flow through the sides of the test tube It is used to perform simple chemical reactions.

2.1.2 Pipet

There are many kinds of Pipets.

(a) *Volumetric pipet:* It is a long narrow glass tube with a bulb in the middle and a mark on the stem. The capacity is marked on the bulb. The capacity ranges between 2,5,10,20 millimetre (c.c). It is used to measure a definite volume of liquid.

While using a pipet, hold it in the right hand, the container in the left hand. Keep the tip of the pipet immersed in liquid column of the container The nozzle is held just above the bottom of the container. Keep the other end between the lips and slowly suck the liquid. The liquid will rise in the pipet. When the liquid column rises a little above the mark, stop sucking Close the end with the index finger Raise the pipet from the liquid. Wipe the tip with filter paper. Apply pressure by raising the finger a little. Bring the lower meniscus to touch the mark In case of coloured solution like blood, upper meniscus should coincide with the mark. This will give the exact amount of the liquid While transferring the liquid, when it is a bulb pipet do not blow the little column of liquid which remains in the pipet. It is used in volumetric estimations and titrations.

(b) *Serological pipet:* It is a very narrow, graduated glass

apparatus. It is used to measure out liquids of small volume i.e., fraction of a millilitre like 0.2 ml etc. It is used strictly for accurate analysis and estimations. While transferring the liquid, the last drop of the liquid must be blown off and collected if it is a blow-out pipet, that is the one which is graduated upto the tip.

(c) *Ostwald pipet:* It is used to deliver a fixed volume like 0.1 ml, 0.2 ml, 0 5 ml etc. Transfer the volume completely in between the tip and the mark.

(d) *Lang levy pipet:* This is of the shape shown in the Fig The volume transferred is between the tip and the constriction seen inside the pipet It is available in different volumes.

Note: After the use the pipet is kept soaked in water. In case of block in the tip, it is kept soaked in chromic acid wash solution for a few hours. Then it is cleaned thoroughly first in tap water then in distilled water before the next use. The dirt blocking the tip can also be removed by inserting a thin wire

2.1.3 Conical Flask

It is a cone shaped glass apparatus with a narrow neck. It is used in volumetric estimation and titration and to boil the liquids also. It is also called Erlenmeyer flasks. It is available in various volumes, like 25 ml, 50 ml, 100 ml, 250 ml etc.

2.1.4 Burette

It is a very narrow glass tube, graduated in millilitre (c.c). One end is provided with a pointed nozzle which is connected to the glass tube by a rubber tube The rubber tube is provided with a pinchcock Rinse the burette with the liquid before. While using, hold the burette in the left hand and pour the liquid directly from the container to the burette. Fill the burette with the liquid above the zero mark. Remove air in the rubber tube by closing the mouth of the tip with thumb and closing and releasing the pinchcock. Air bubble rises up. When the air bubble ceases to rise up, adjust the liquid column to zero mark, Now it is ready for use. Always take base in burette.

2.1.5. China Dish

It is a hemispherical, shallow porcelein vessel with a small spout. It is used in evaporation and other boiling processes.

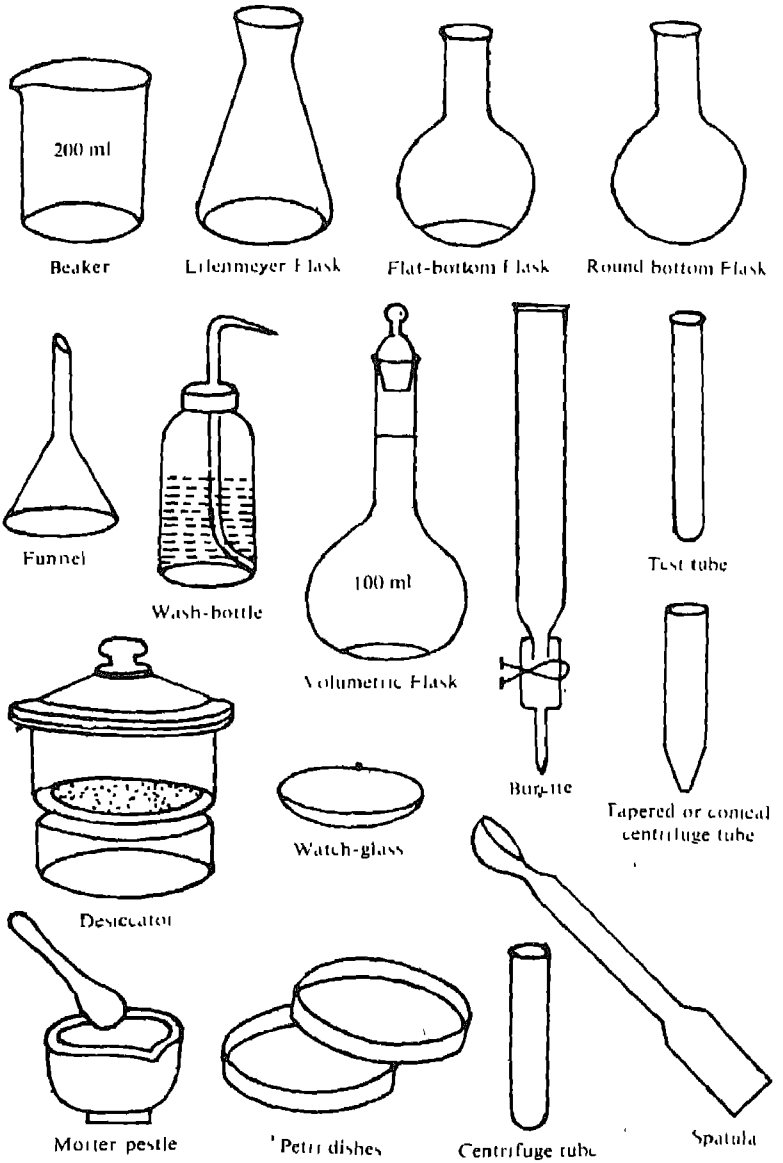
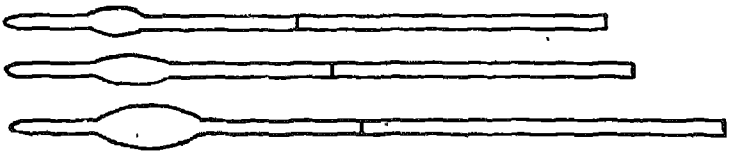
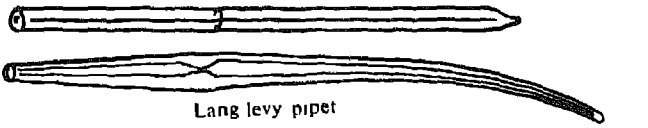


Fig VII-1 LABORATORY GLASSWARE

A



Ostwald pipet



Lang levy pipet

Volumetric (bulb) pipet

Serological pipet
(blow out type)

Pasteur pipet

Graduated cylinder

2.1.6 Beaker

It is a cylindrical glass vessel with a small spout. It is used to take and transfer the liquids. It is also used in volumetric estimations and preparations of liquids. Capacity ranges from 5 ml to a few litres.

2.1.7. Graduated Cylinder (Measuring Cylinder)

It is a modified form of beaker, graduated in c.cs or mls. The capacity ranges from 10 c.c. to 2 litres. It is used to measure a rough amount of liquid in preparing solutions or reagents. It is available as stoppered and non-stoppered graduated cylinder.

2.1.8. Spatula

It is made of plastic or steel. One end is spoon like to take and transfer the solids. The other end is sharp and flattened used for cutting the solids.

2.1.9. Volumetric Flask

It has a long narrow neck and cone shaped body with a flat bottom. It is used to make up a solution into a definite known volume. There are measuring flasks of capacity 10 ml, 50 ml, 100 ml, 250 ml, 1 litre etc.

2.1.10. Funnel

It is a glass apparatus used to transfer and filter the liquids. It is cone shaped with a thin long stalk.

2.1.11. Centrifuge Tube

(a) It is a thick walled narrow glass tube with one end closed. It is used in the separation techniques. The weight of two centrifuge tubes must be matched exactly. It is convenient to properly label the pairs of exactly matching tubes with the help of diamond pencils.

(b) *Tapered, Stoppered Centrifuge tubes:* This is similar to the above one. But the closed end is tapered and the outer end is provided with stopper. It is used in separation technique specially when organic solvents are involved.

2.1.12 Watch-Glass

It is a shallow glass plate. It is used to weigh solids and to cover during boiling. It is available in various sizes.

2.1.13 Petri Dish

It is like a beaker cut to a height of 1 75 to 2 5 cm from the bottom. It is used to wash the electrophoresis strip in solutions and also for other purposes

2.1.14 Desiccator

It is of the shape shown in the figure The lower compartment is filled with anhydrous calcium chloride or silica (dried). A perforated porcelein plate is placed over. Substance to be kept dry is placed over this porcelein plate. Grease is smeared all over the flat edge of the body and the lid The lid is placed in order. When placing or removing the lid slide over the lid to one side and lift.

Change the desiccating material that is $Cacl_2$ when it becomes yellow or silica when it becomes pink or red Used up silica (pink or red) can be reused after drying it in hot-air-oven at 120°C till it regains the blue color

2.1.15 Wash-Bottle

It is made of polythene or glass The tubing should touch the bottom or at least a little above as shown in Figure This is used to transfer distilled water

2.1.16 Pestle-Mortar

This is used to powder or grind substance to prepare a paste

2.2 Basic Techniques

2.2.1 Introduction

Biochemical analysis demands great accuracy as the constituents in biological fluids are in minute quantities The accuracy is attained only when one is well versed with the basic techniques like pipetting, weighing, etc.

2.2.2 Methods of Measuring Liquids

The measuring glasswares most frequently used are graduated cylinders, volumetric flasks and pipets

(a) *Units of Measurements*· Most commonly the measurement is made in litres or its fractions In metric system deci is 1/10th, centi is 1/100th, milli is 1/1000th and μL is 1/1000th of 1/1000 i.e. 1/10,00,000, so that 1 L=10 dl=100 cL=1000 mL=10,00,000 μl

i e , 1 dl = 1/10th L, 1 ml = 1/1000th of a litre and 1 ml = 1000 μl (microlitre) See Table VII-C Table of Units

(b) *Graduated Cylinders:* Volumes above 25 ml are often measured using graduated cylinders. While using the graduated cylinder lower meniscus of the liquid column in the cylinder is made to coincide with the definite mark on the cylinder. Consider the upper meniscus if it is a coloured fluid

But volumetric flasks are preferred when it is desired to transfer fixed volumes like 25 ml, 50 ml, 100 ml, 250 ml, 500 ml etc. It is remembered that the solution required to be measured is always allowed to attain room temperature before measuring because liquids expand to heat and contract to cold temperature.

(c) *Pipets*· The use of pipet is very important in clinical analysis, if the pipetting has been inaccurate the result is worth nothing. So correct method of pipetting is very important to get accurate results. There are serological, volumetric or bulb pipet and ostwald pipets.

While transferring fluids using pipets if it is a pipet graduated upto the tip i e., 'blow-out' type pipet, the last portion of fluid in the

Table VII. U Table of Units

(a) Weight:			
	1 Kg	=	1000 gms
	1 gm	=	1000 mgs
	1 mg	=	1000 μg (microgram)
	1 μg	=	1000 ng (nanogram)
	1 mg	=	10^{-3} gms
	1 μg	=	10^{-6} gms
	1 ng	=	10^{-9} gms
(b) Volume:			
	1 L	=	1000 ml
	1 ml	=	1000 μl (microlitre)
	1 ml	=	10^{-3} L
	1 μl	=	10^{-6} L
Note. 1 c.c = 1 0004 ml (Approx) But for practical purposes 1 ml = 1 c.c			
(c) Length:			
	1 m	=	100 cm
	1 cm	=	10 mm
	1 mm	=	1000 μm (micrometer)
	1 μ	=	1000 mμ (millimicrons)
	(1 micron)		
	1 mμ	=	1 nm (nanometer)
	1 nm	=	10 A (Angstrom units)
(d) Strength of Solution:			
	1 M	=	1000 mM (millimolar)
	0.1 M	=	100 mM
	0.01 M	=	10 mM
	0 001 M	=	1 mM
	1 mM	=	1000 μM (micromoles)
	1 Eq/L	=	1000 mEq/L
	1 mEq/L	=	mgs per dl × 10 ÷ Eq. wt

pipet is delivered into the container, otherwise upto the lowest graduation on it, if it is not a blow out type. When using a bulb or volumetric pipet the portion of the fluid in the tip or nozzle is not collected In these instances the fluid is allowed to drain by itself with the tip of the pipet touching the bottom or the wall of the container.

Ostwald pipets are used to deliver viscous fluids like blood, serum or plasma usually in fixed volume less than an ml In this case the portion of fluid in the tip is delivered and collected.

It is important to bear in mind that the meniscus level is

observed holding the pipet vertical and line of vision in line parallel to the ground Otherwise there is the error of parallax

2.2.3 Cleaning of Glasswares

The glasswares are brushed well with some detergents like vim or liquid detergent like Teepol They are washed thoroughly in running tap water 3 or 4 times followed by rinsing twice or thrice with distilled water. The cleaned glasswares, excepting the graduated glasswares, are dried in hot-air-oven

Pipet after constant use may have greasy adherents on the walls indicated by air bubbles in the liquid column inside. In such cases or generally once in a week the pipets are washed in chromic acid. Glasswares are kept dipped completely in chromic acid for a few hours and they are washed under running tap water thoroughly followed by three or four rinsings in distilled water The cleaned pipets are kept inverted over a pad of dry filter paper vertically on a pipet stand. The glasswares are now ready for use

Chromic acid wash solution

(a) Prepare a saturated solution of potassium dichromate.— Take about 200 ml water Add potassium dichromate crystals with brisk, continuous stirring till some crystals remain undissolved Store in a bottle

(b) Concentrated sulfuric acid, LR grade:— Now transfer 25 ml of saturated dichromate solution to a 2-litre beaker kept in a sink with a continuous flow of tap-water outside for cooling Add slowly and carefully one litre of concentrated H_2SO_4. Cool Transfer to a tall glass jar. The solution is very corrosive. Handle carefully or use hand gloves. The solution is red in colour After continuous use it becomes green which is then useless for cleaning Replace with fresh preparation It is advisable to keep chromic acid near the sink for purposes of safety.

2.2.4 Method of Weighing

The rough balance and the analytical balance are used to weigh substances (Fig. VII 2) Rough balance is not sensitive whereas analytical balance gives accurate weight correct to fourth decimal place of a gram. So the use of analytical balance requires good technique. Some precautions are taken to make correct weighings.

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11 Rider support
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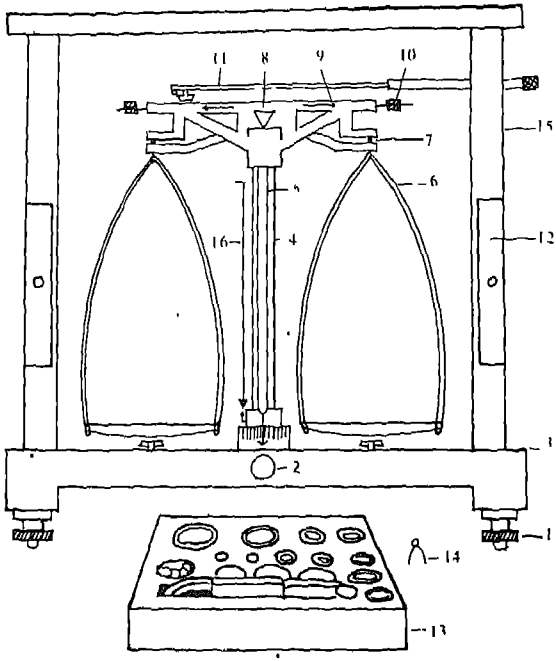
14 Rider
15 Glass case



Rider scale on the beam-magnified

Fig VII-2 ANALYTICAL BALANCE

With each analytical balance there is a set of weights The weights are well protected from dust and moisture Thus the weights should be handled carefully with the help of forceps The weights are placed on the right-hand pan. The weight box is provided with a loop of wire of the shape 'λ' This is meant for use on the rider scale The use of rider makes possible to weigh objects correct to fourth decimal place of a gram On the rider scale each mgram is divided into 10, each portion corresponds to 0.1 mg For e g. 5 2121 gms means the weights used are 5 gms, 200 mgs, 10 mgs and the last two digits 2 1 mgs, so the rider is placed on the first tooth after the digit '2' on the rider scale.

In weighing 5.2121 gms the following weights are used:

Gram weights	*mgm weights*
5 gms	200 mgs
	10 mgs

Rider scale: 1st division after '2' mg.

This is a sensitive balance The instrument should be placed where there is controlled temperature (possibly air-conditioned room) and there are no wind drifts, corroding fumes, dust etc or vibrations.

Before operation study the parts of the analytical balance. During the procedure of weighing observe the following precautions:

1 Always handle the weights with a pair of forceps.
2. Never add or remove anything off the pans unless the balance is at rest. This protects the sharpness of the agate knife edges and hence sensitivity of the balance.
3 Do not give a jerky movement to the knob
4. Do not weigh substances unless it comes to room temperature.
5 Before weighing see that the platform is horizontal indicated by index (work on lavelling screws to make horizontal)
6. The pointer should show equal oscillations on either side of 'O' on the scale; otherwise work on terminal adjustment screws and make the weights of empty pans balance exactly. All determinations in biological fluids are worthless if weighing and pipetting are not done accurately besides following conditions of test strictly

2.2.5 Separation of Solids from Liquids

This is done either by filtration or centrifugation.

Filter paper is used for filtration Whatman filter papers are available with different pore sizes and hence using which the rates of filtrations are different.

Select a circular filter paper or cut into a circle Fold it twice to obtain a cone shape Place into the funnel. Pour the fluid through a glass-rod into the funnel. Collect the clear filtrate in dry vessels

Ordinary filter paper serves the purpose in most cases with thick precipitates. If the precipitate is in suspension or gelatinous, select a whatman filter paper There are various grades of filter papers as given in the Table VII-2.

Table VII-2. Grades of Filter Paper

Filter speed	*Whatman No*	*Separates*
Fast	4	Coarse and gelatinous precipitates
	54	
Medium fast	1	Medium crystalline suspensions
	43	
Medium	2	Crystalline suspensions
	40	
	52	
Slow	5	
	6	
	42	Fine crystalline suspensions
	44	
	50	

Centrifuging is done using a table centrifuge. Here it is very important that the two arms (opposite) are balanced exactly. Centrifuge tubes are weighed and placed in the two buckets only when the weights of these are exactly same; otherwise the centrifuge will be spoiled To ensure this the test fluid is taken in a centrifuge tube and the weight of this is exactly made equal to another centrifuge tube with water. These two tubes are placed in two buckets so that they are directly opposite each other in the centrifuge. This balances the centrifuge Slowly turn the operating knob Do not give maximum speed at once. Adjust to the required RPM (Rotations per minute). Usually centrifuge for 5 to 10 minutes. If the fluid is viscous centrifuge for 15 minutes When the tubes stop spinning, remove them from

their holders The precipitate is in bottom of the tube, pour off the supernatant If the supernatant is used for further determinations pour in another tube and transfer the required volume using a pipet.

Note: Replace the carbon brush when worn out with a new one. This is indicated by slackening in the speed of rotation

2.3 Chemicals and Reagents

2.3.1 Grades of Chemicals

Chemicals are available in different grades of purity A Laboratory Reagent grade (LR) chemical is less pure Whereas Analar or Analytical Reagent (AR) grade and GR or Guaranteed Reagent are high purity chemicals. AR or GR grade chemicals are used wherever they are mentioned; instead, if LR chemicals are used an erroneous result is got AR and GR grade chemical are equal in purity.

2.3.2 Common Chemicals and Their Preservation

Knowledge of preservation of chemicals is imperative A list of common chemicals and their preservation is given in Table VII-3

Table VII-3. Common Chemicals and their Preservation

	Name of Chemical	*Preservation*
1	Agar	Shelf
2.	Alpha-ketoglutaric acid	at 40°C, refrigerator
3.	Amido Schwartz 10B	Shelf
4.	1,2,4,—Aminonaphthol—sulfonic acid	Shelf
5.	4—Aminoantipyrin	Desiccator
6	Ammonium molybdate	Shelf
7.	Barbitone	Shelf
8	Barium chloride	Shelf
9.	Barium hydroxide	Shelf
10	Benzidine	Shelf
11.	Benzoic acid	Shelf
12	Borax	Shelf
13	Bilirubin	Dark or in refrigerator.
14	Bovine albumin	Shelf
15	Bromine	Shelf, tightly stoppered
16.	Calcium carbonate, anhydrous	Shelf
17.	Calcium chloride, anhydrous	Shelf
18.	Calcium chloride, fused	Shelf
19.	Chlorofom	Shelf, tightly stoppered

20	Cholesterol	Shelf
21.	Citric acid	Shelf
22	Copper sulphate	Shelf
23	Creatinine	Refrigerator (4°C)
24	O-Cresolphthalein complexone	Shelf
25.	Diacetyl monoxime	Shelf
26.	Dimethryl aminoazobenzene	Shelf
27	2,4,—dinitrophenyl hydrazine	Shelf
28	Diphenyl carbazone	In dark
29	Dipotassium hydrogen phosphate	Shelf
30	Disodium EDTA (Ethylene diamine tetra acetic acid, disodium salt)	Shelf
31.	Disodium hydrogen phosphate	Shelf
32.	Disodium phenyl phosphate	Shelf
33.	DL-Aspartic acid	Shelf
34	EDTA	Shelf
35	Ethanol	Shelf
36.	Ethanolamine	Shelf
37	Ethylether	Tightly stoppered away from flame
38.	Ferric chloride	Desiccator
39	Ferric nitrate	Desiccator
40.	Ferrous ammonium sulphate, hexahydrate	Shelf
41	Formalin (Formaldehyde)	Shelf
42.	Glacial acetic acid	Shelf
43.	Glusoce (dextrose)	Shelf
44.	Horse gram powder	Shelf
45.	Hydrochloric acid	Shelf, tightly stoppered
46.	Hydrogen peroxide	at 4°C in refrigerator
47.	8-Hydroxyquinoline	Shelf
48.	Indicator paper (various ranges)	Shelf
49.	Iodine	Shelf
50.	L-Alanine	Shelf
51	Lithium carbonate	Shelf
52.	Lithium sulphate	Shelf
53	Litmus paper blue and red	Shelf
54.	Liqour ammonia	Shelf
55	Mercuric chloride	Shelf
56.	Mercuric nitrate	Shelf
57	Merthiolate	Shelf
58.	Methanol	Shelf
59.	Naphthyl hydroxamic acid	Shelf
60.	Nitric acid	Shelf, tightly stoppered
61	Ortho-phosphoric acid	Shelf
62	Ortho-Toluidine	Shelf (preferably in dark)
63.	p-dimethyl aminobenzaldehyde	Shelf
64	Petroleum Ether	Tightly stoppered away from flame
65	Phenol	Shelf
66.	Phenolophthalein	Shelf

67.	Phenyl mercuric acetate	Shelf
68	Picric acid	Shelf
69	Phosphoric acid	Shelf
70	Potassium chloride	Shelf
71	Potassium dichromate	Shelf
72	Potassium dihydrogen phosphate	Shelf
73.	Potassium ferricyanide	Shelf
74	Potassium hydroxide	Shelf
75.	Potassium iodide	Desiccator
76	Potassium oxalate	Shelf
77	Potassium permanganate	Shelf
78	Potassium thiocyanate	Shelf
79	Silver nitrate	Shelf
80.	Sodium acetate	Shelf
81	Sodium azide	Shelf
82	Sodium barbitone	Shelf
83.	Sodium benzoate	Shelf
84.	Sodium bicarbonate	Shelf
85	Sodium carbonate, anhydrous	Shelf Room Temp
86.	Sodium chloride	Shelf
87	Sodium citrate	Shelf
88	Sodium dihydrogen phosphate	Shelf
89	Sodium disulphite or Sodium metabisulphite	Shelf
90.	Sodium fluoride	Shelf
91	Sodium hydroxide	Shelf
92	Sodium molybdate	Shelf
93	Sodium nitrite	Shelf
94	Sodium nitroprusside	Shelf
95	Sodium potassium tartrate	Shelf
96.	Sodium pyruvate	at 4°C, refrigerator
97	Sodium sulfate	Shelf
98	Sodium sulfite, anhydrous	Shelf
99	Sodium thiosulphate	Shelf
100.	Sodium tungstate	Shelf
101.	Starch, soluble, AR Extra pure	Shelf or desiccator
102	Sulfanilic acid	Shelf
103.	Sulfosalicylic acid	Shelf
104.	Sulfuric acid	Shelf
105.	Sulphur powder	Shelf
106	Tartaric acid	Shelf
107	Thiosemicarbazide	Shelf
108.	Thiourea	Shelf
109	Trichloro acetic acid	Desiccator
110	Tris-(Hydroxymethyl) aminomethane	Shelf
111.	Tri-sodium citrate	Shelf
112	Urea	Shelf
113.	Uric acid	Shelf
114	Zinc sulphate	Shelf

2.3.3 Preparation of Solutions

The following types of solutions are prepared in clinical laboratory.

1. Saturated solution
2. Percent solution
3. Molar solution
4. Molal solution
5. Normal solution

2 3.3.1 Saturated solution:

When a solid is dissolved in a liquid the solid is called as the solute and the liquid is called as the solvent.

A saturated solution is one which holds as much solute it can.

Eg. Preparation of saturateed solution of sodium chloride.

About 100 ml of water is taken in a beaker. Sodium chloride is added in small portions to this with constant stirring. Continued the addition till some crystals are lef undissolved. Now the solution is saturated with sodium chloride, filled in a bottle and added one more spatula of the salt The bottle is labelled as "saturated sodium chloride solution" with date of preparation and name of the person prepared

2.3.3.2 Percent solution:

A percent solution is one which contains a known weight of the substance in a specified volume of its solution. If the solute is a solid when it is percent solution wt/vol and if it is a liquid then it is vol/vol percent solution.

Eg: Preparation of 5% sodium chloride solution.

Here 5 gms of NaCl are contained in 100 ml of its solution. To prepare it, about 70 ml of water is taken in a beaker, exactly 5 gms of sodium chloride are added to it. It is completely dissolved. Transferred to a 100 ml volumetric flask or in a measuring cylinder with stopper The vol. in the cylinder is made upto mark with distilled water. The cylinder is stoppered, inverted and swirled several times for uniform mixing. It is then filled to a reagent bottle and labelled properly.

A percent solution Volume/Volume is prepared as follows.

Eg: Preparation of 5% HCl solution.

A little distilled water, say, 40-50 ml is taken in a 100 ml standard flask. A 10 ml pipet is taken and fixed a rubber teat to the sucking

end. Concentrated HCl is drawn into the pipet with the help of the teat. Exactly 5 ml acid is transferred to the flask containing water with constant swirling. Then water is added upto the mark. The flask is stoppered, inverted and swirled well for uniformity of solution. It is then transferred to a reagent bottle and labelled.

Note: Always add *acid to water* with mixing. This is important because if water is added to acid, water spirts generating great quantity of heat causing the acid to splash out. Moreover, when concentrated acids, especially sulfuric acid is diluted it is done under cold, i e , in an ice chest containing ice cubes Addition of acid is done in small amounts with constant stirring.

Exercise:

1. Prepare 100 ml of normal saline i.e 0.875 gms%
2 Prepare 10% sulfuric acid solution (run tap water outside the the beaker while diluting)

Note: 1. Add acid to water.
2 Keep on mixing during additon.
3. If more than 20% sulfuric acid is required the dilution is done under cold water or ice cubes.

2 3.3 3 Molar solution·

A molar solution is one, a litre solution of which contains 1 gm molecular weight of the substance. It is denoted as '1 M' solution. When the weight of the solute dissolved in one litre of its solution is equal to its molecular weight it represents 1 M solution.

Molecular weight of a substance is obtained by adding the atomic weights of the elements in the proporation contained in the compound

Eg· NaCl

Atomic weight of Na = 23
Atomic weight of Cl = 35.5
: Molecular weight of NaCl = 58 5

H_2SO_4

Atomic weight of H = 1
Atomic weight of S = 32
Atomic weight of 0 = 16
. Molecular weight of H_2SO_4
$= 1 \times 2 + 32 \times 1 + 16 \times 4$
$= 2 + 32 + 64$
$= 98.$

If the molecule of a compound is hydrated, the weight of water is also summed up to the molecular weight of the compound

Eg $COOH\text{-}COOH\ 2H_2O$ i e., $C_2H_2O_4\ 2H_2O$

Molecular weight = $12 \times 2 + 1 \times 2 + 16 \times 4 + 2(1 \times 2 + 16)$

$= 24 + 2 + 64 + 36$

$= 126$

:. Mol. wt of Oxalic acid = 126

When the weight in grams taken is equal to its molecular weight it is called gram-molecular weight. 1 molar solution is also called as 1 gram-mole or 1 mole.

Preparation of 1 M sodium chloride solution:

Since a molar solution contains 1 g molecular weight per litre, we must find to exact weight to be taken for the preparation of known volume This is found in the following way

Weight to be taken = $M \times S \times V$

where M = Molecular weight

S = Strength of required solution, i e., say, 0.1 M, 0.2 M. 0.3 M etc.

V = Volume in litres i e., 1/10, 1/4 or 1/5 or 1 L.

So to prepare 100 ml of 1 M sodium chloride solution

$wt = M \times S \times V$

$= 58.5 \times 1 \times 1/10 = 5\ 85$ gms

Method. Weigh out a clean and dry watch glass. Add 5.85 gms weight on right pan. Now add sodium chloride crystals till the pointer shows equal oscillations on either side of zero of the scale Transfer the weighed crystals carefully and completely into a 100 ml standard flask through a funnel using distilled water. Dissolve the crystals completely. Add water upto 3/4ths, mix. Then add water upto the mark. Stopper the flask, and swirl well for uniform mixing. Transfer the solution to a clean reagent bottle Label it as IM sodium chloride with date and initials.

Exercise Prepare 100 ml of 0.2 M sodium carbonate solution (molecular weight is given on the bottle) Using the above stock, prepare 100 ml each of 0.1 M and 0.02 M solution

Note. For dilution of 0.2 M to get 0.1 M, dilute the stock 1 1 with water or 1 in 2 with water. 1:1 dilution is expressed as 1 in 2 dilution.

i e., dilute 50 ml of 0.2 M solution to 100 ml in a 100 ml volumetric flask.

For 0.02 M solution, dilute the stock 1 in 10 with distilled water, i e., 10 ml of 0 2 M solution is diluted to 100 ml in a 100 ml standard flask

2.3.3 4 Molal solution·

A molal solution is one which is prepared by dissolving one gram-molecular weight of a substance in one thousand grams of its solvent.

Eg. 58.5 g. sodium chloride dissolved in 1000 g of water to get 1 molal solution of sodium chloride solution. This type of solution is seldom used

2.3.3 5 Normal solutions:

Normal solutions are used extensively in the laboratory The word normal is denoted by the letter 'N'. So one normal solution is denoted as 1 N, 2 normal as 2 N and N/100 as 0.01 N solutions etc

Definition of a normal solution:

A normal solution is one which contains one gram equivalent wt. of solute dissolved in one litre of its solution.

Eg: If the equivalent weight of a substance is 20 then 20 gms is the gram equivalent wt. When 20 gms of that is dissolved in a little of water and then made up the volume to 1 litre it is 1 N solution. Likewise 40 gms of that substance in 1 L, 10 gms of it in 1 L and 2 gms of it in 1 L respectively are 2 N, 0.5 N and 0.1 N solutions.

So to prepare a normal solution equivalent weight is calculated as follows.

Equivalent weight of an Acid:—Acid is one which has ionisable or replaceable hydrogen ions (H) If an acid has one replaceable H^+ it **is called a monobasic acid, if 2 it is called a dibasic acid, and 3 it is a** tribasic acid. A monobasic acid forms one type of salt, dibasic two types and tribasic acid forms three types of salts on treating with base.

Eg: HCl forms only NaCl. So it is monobasic.

H_2SO_4 forms $NaHSO_4$ and Na_2SO_4 and so it is dibasic

$$\text{Equivalent weight of an acid} = \frac{\text{Molecular weight}}{\text{No. of replaceable } H^+}$$

Eg: Equivalent Wt. of HCl $= 36.5 = 36.5$

i.e., for monobasic acids, molecular weight = eq. wt. (Equivalent weight)

Hence 1 M solution = 1 N solution.

Whereas eq. wt. of H_2SO_4 = $\frac{\text{Molecular weight}}{2}$

$$= \frac{98}{2} = 49$$

i.e. 1 M H_2SO_4 = 2 N

Likewise equivalent weight of H_3PO_4 = $\frac{\text{Molecular weight}}{3}$

i.e , 1 M H_3PO_4 = 3 N H_3PO_4

Now to find the weight to be taken for a known volume of V litres of acid of strength 'N' of equivalent weight 'E',

Wt. for V litres of N Normal = E × N × V gms.

Equivalent weight of a base is molecular weight divided by the number of replaceable hydroxyl ions Eg:— NaOH has one replaceable OH ion and hence, equivalent weight of

$$NaOH = \frac{\text{Mol. wt}}{1} = \frac{40}{1}$$

Calcium hydroxide $Ca(OH)_2$ has 2 replaceable hydroxyl ions and its equivalent weight

$$= \frac{\text{Mol. wt.}}{2}$$

$$= \frac{74}{2} = 37$$

Now, equivalent weights of salts such as $AgNO_3$ and $KMnO_4$ & $K_2Cr_2O_7$ are determined by the number of electrons which they give or take during a reaction.

Eg. $AgNO_3$ gives 1 electron Therefore equivalent weight of $AgNO_3$ is molecular weight divided by 1

Potassium permanganate takes 5 electrons, therefore the equivalent weight is molecular weight divided by 5. Same way equivalent weight of $K_2Cr_2O_7$ is molecular weight divided by 3.

Table VII-4 Equivalent weights of some common chemicals

Compound	*Mol wt*	*Eq. wt*	*% w/w*
Acetic acid, glacial	60.05	60 05	100
Hydrochloric acid	36.461	36 461	37
Sulphuric acid	98 078	49.039	96
Oxalic acid	126 067	63.033	
Sodium hydroxide	40 000	40 000	
Sodium carbonate	106 000	53 000	
Potassium permanganate	158 038	31 607	
Nitric acid	63 03	63 03	70

Preparations of normal solutions.

Exact normal solutions can be prepared by weight or by dilution only when a chemical is available in its purest state. Moreover correct weighing is possible if chemical does not absorb or lose water on exposure. On the other hand, liquids like acids are not pure as supplied commercially and so an exact solution is possible to prepare by dilution.

Sodium carbonate and oxalic acid are available in the pure form and so an exact solution of these is possible to prepare by weight These are called primary standards. Sodium hydroxide liquifies on exposure by absorbing moisture. That being the case an exact sodium hydroxide solution cannot be prepared by weight. Sodium carbonate can be prepared and used as a primary base whereas oxalic is a primary acid, using which other solutions are standardised. Standardisation is done by titration which will be dealt later

Preparation of primary standard sodium carbonate solution

Eg To prepare 100 ml 0.1 N sodium carbonate solution

Molecular weight of sodium carbonate = 106

$$\text{Equivalent weight of sodium carbonate} = \frac{106}{2}$$

$$\therefore \text{Weight in gm for 0 1 N, 100 ml solution} = E \times N \times V$$

$$= \frac{0.1 \times 53 \times 100}{1000}$$

$$= 0\ 53 \text{ gms}$$

Method. Make the preliminary adjustments in an analytical balance. Take a clean and dry watch glass and place on the left hand pan Place weights in the descending order on the right hand pan checking

now and then, and finally note the exact weight of the watch glass. Now place 530 mgs weight on the right pan. Add sodium carbonate crystals a little by little till the weights of two pans match exactly This is indicated by the pointer by its oscillations equally on either side of midpoint on the scale.

Now transfer carefully the weighed crystals into a clean 100 ml standard flask. Use a clean funnel Wash down the crystals completely into the flask with a jet of distilled water Remove the funnel Swirl the flask to dissolve the chemical. Add distilled water, when it is filled to 3/4ths, swirl again. Then add water upto the mark Stopper the flask, invert and swirl well for complete mixing Now this is a decinormal sodium carbonate solutions Transfer to a clean reagent bottle and lable as "O.1 N Sodium carbonate sol " with date and initials

Exercise· Prepare 100 ml decinormal oxalic acid solution

Mol. wt. of oxalic acid, COOH-COOH 2 H_2O = 126 067

Equivalent wt of Oxalic acid = 126 067/2

= 63.033

Weight for 100 ml 0.1 N Solution = ESV/1000

$$= \frac{63\ 033 \times 0.1 \times 100}{1000}$$

$$= 0.6303$$

Accurately 0.6303 gm oxalic acid crystals are weighed and prepared 100 ml solution as is done in the previous expt. and filled in a clean reagent bottle and labled

2.3.4 Acids and Bases

Acids liberate hydrogen ions (H^+) in solution They are the H^+ donors Bronsted defined acids as proton donors and bases as proton acceptors. Acids change blue litmus to red and bases change red litmus to blue.

Strong and weak acids· Acids are said as strong or weak on the basis of extent of ionisation in solution.

Ionisation is the phenomenon of splitting up of molecular into charged particles in solution

Eg: HCL ionises as H^+ and Cl^- (chloride ion). This is reversible and written as

$HCl \rightleftharpoons H^+ + Cl^-$

Mineral acids like acids HCl, HNO_3 & H_2SO_4 ionise in solution to 90—95% whereas organic acids like acetic acid and oxalic acid ionise to less than 10%. So HCl, HNO_3 & H_2SO_4 are called strong acids while acetic and oxalic acids are weak acids.

The same classification holds good with bases also. Sodium hydroxide is a strong base whereas sodium carbonate is a weak base

2.3.5 Chemical Indicators

There are certain chemicals which are used as indicators to acid-base titration These chemicals may be weak organic acids or bases or dye stuffs which change color as the hydrogen ion concentrations in solution increase or decrease. The reason for change of color is reversible ionisation of these indicator molecules. The ionised particles will have one color and the unionised molecule of it have an entirely different color. Ionisation of different indicators takes place at different pH ranges

Eg. Phenolphthalein is a colorless molecule, when it ionises it is pink. This takes place between a pH of 8.3-10.00 So below pH 8.3 it is colorless Beyond 8 3 up to 10 ionisation goes on and different shades of pink color are obtained.

Beyond pH 10 no change in pink is observed So pH 8.3-10 0 is called the effective pH range of phenolphthalein

Table VII-5 List of indicators, their characteristics & Preparation

Name	*PH range*	*Color change*	*Preparation*
Thymol Blue Ist change	1 2 to 2.8	Red-Yellow	0.1g, 4 3 ml 0 05N NaoH to 250 ml with water
Topfer's reagent (p-Dimethylaminoazo-benzene)	2.9 to 4.2	Red-Yellow	0 5g in 100 ml 95% alcohol
Methyl orange	3 0 to 4 4	Red-Yellow	0.1g in 100 ml water
Bromocresol Green	3 8 to 5 4 3 3	Yellow-Green	0.1g, 2 9 ml 0 05 N, NaOH, to 250 ml with water
Phenolphthalein	8.3 to 10.0	Colorless-Pink	0 1g to 1g% in 50% achcohol

2.3.6 pH and pH Scale

Acids ionise in solution to liberate H^+ A solution is said more acidic when hydrogen-ion concentration (H^+) is more. This prompted the earlier scientists to devise a method for the measurement of (H^+).

The hydrogen-ion concentration (H^+) in solution is very small like $\frac{1}{10}, \frac{1}{100}, \frac{1}{1000}, \frac{1}{10,000}$ etc gram moles per litre. One litre water has 10^{-7} gm moles per litre of (H^+), so pH of pure water is said to be 7. The integer 7 is got by retaining only the exponent of 10, the negative sign being dropped. Accordingly pH $1 = 10^{-1}$ gm moles per litre (H^+), pH $2 = 10^{-2}$ gm moles per litre of (H^+), etc.

pH also is defined as the Logarithm of the reciprocal of (H^+) i.e , $pH = \text{Log } \frac{1}{(H^+)}$.

Sorenson gave the pH scale which ranges from 0 to 14, pH 0-7 is acid scale and pH 7-14 basic scale and pH 7 is neutral Thus the acidity of solution of pH 0 > pH 1 > pH 2 > pH 3 > pH 4 > pH 5 > pH 6 > pH 7.

And alkalinity of pH 14 > pH 13 > pH 12 > pH 11 > pH 10 > pH 9 > pH 8 > pH 7

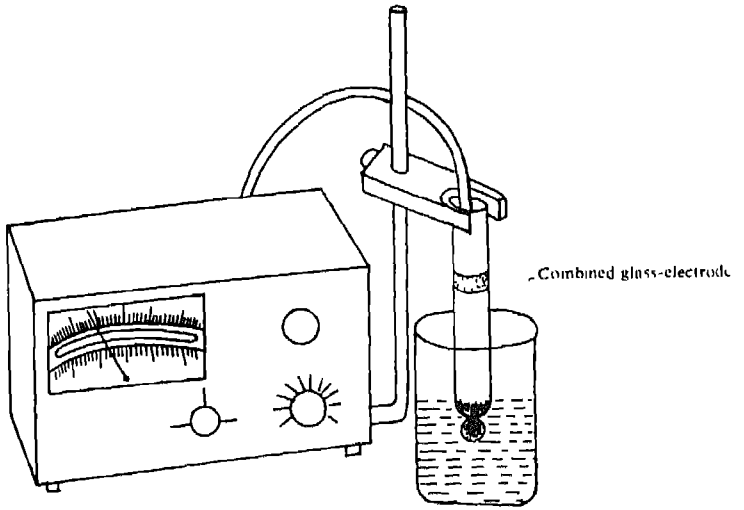


Fig. VII-3 pH METER

2.3.7 Measurement of pH of Solutions

Using different indicators the approximate pH of unknown solution can be found. Indicator papers of various pH ranges are available, using which the pH of unknown solutions can be found

Dip a piece of indicator paper in unknown solutions. Compare the color of this with those given on the book of indicator paper

To get a precise measurement of pH use a pH meter. A pH meter consists of a combined glass electrode. The electrode is always kept immersed in water A needle moves over the scale marked from 0-7 and 7-14. The instrument is standardised with the standard buffer It is calibrated with the standard buffer of pH near that of unknown Eg Say, a solution has a pH around 4. The instrument is calibrated with the standard buffer of pH 4 0 or any buffer nearer pH 4.0 If another is around 8, then the instrument is calibrated with standard buffer of pH 8 or any other standard buffer near pH 8.

Procedure for the measurement of pH of solution· Say the pH of Test solution is around 9 Put on the switch, wait for 10 minutes. Take out the electrode from water by moving up the electrode, wash the outside of electrode with a jet of water. Turn the knob 'set zero' and set the needle to Zero. Dip the electrode into the standard buffer of pH 9 taken in a beaker Turn the knob 'standardise' and set the needle to '9'. Take out the electrode from the solution. Wash the electrode with a jet of water. Take test solution in a beaker Dip the electrode into the test solution Read the pH on the scale. Remove the electrode from Test solution. Wash the electrode with a jet of water. Keep it dipped in water.

Note:

1. The electrode is made of thin film of glass. Handle very carefully.
2 Electrode is always kept wet keeping it dipped in water.

2.3.8 Titrations

2.3.8.1 Neutralising Power of Acids and Bases

The neutralising power of 1N solution is exactly equal to the neutralising power of 1 N base solution. Thus 10 ml of 1 N HCl will exactly neutralise 10 ml of 0.1 N NaOH or 8 ml of 0.1 N HCl will exactly neutralise 8 ml of O. 1 N NaOH. Thus neutralising ability of acids and bases enable to determine the exact normality of solutions (of unknown normality).

For example, suppose we have a hydrochloric acid solution of unknown normality and the problem is to find the exact normality of this solution. The following steps are necessary

Step 1. Preparation of primary standard base.

Step 2· Titration.

Step 3 Calculation.

Method: Exactly 0.53 gms of sodium carbonate is weighed in an analytical balance The weighed crystals are transferred carefully to a 100 ml volumetric flask through a funnel using distilled water It is made upto the mark, swirled and labled. A burette is taken. It is rinsed with standard sodium carbonate solution Then it is filled to zero after removing air bubbles, if any A clean conical flask is taken and rinsed with distilled water. A clean 10 ml pipette is rinsed with the given HCl solution Exactly 10 ml of the acid is pipetted out into the conical flask. A few drops of methyl orange indicator is added (because the acid is a strong acid) and mixed Now standard base is added from the burette carefully The flask is kept rotating during the addition Note the color change When the color change is fast addition of base is made dropwise The appearance of pale orange color denotes the completion of reaction It is end point or neutralisation point Stopped addition of base Noted the burette reading Conical flask is thoroughly cleaned, the titration is repeated till a concordant value is obtained.

Calculation: Volume of acid used = 10 ml
Normality of base = 0.1 N

The values are tabulated as follows (Table VII-6).

Table VII.6 Burette reading (Tabulation)

Trail No.	*Initial burette reading*	*Final burette reading*	*Vol of base added*
1	0	20	20
2	0	20	20
3	—	—	—

Now, $V_1N_1 = V_2N_2$

$10 \times N_1 = 20 \times 0.1$

$$. N_1 = \frac{20 \times 0.1}{10} = 0.2$$

' Therefore, the normality of HCl = 0 2

Table VII-7. Approximate normality and molarity and % of common acids

Acid	*App. Molar*	*App Normal*	*% w/w*
HCl Concentrated Hydroelectric Acid	12	12	37
H_2SO_4 Concentrated sulfuric acid	18	36	96
HNO_3 Concentrated nitric acid	16	16	70
H_3PO_4 Concentrated phosphoric acid	15	45	85
CH_3 COOH Acetic acid, glacial	17	17	100

2 3.8 2 Preparation of 100 ml decinormal HCl solution

Outline:

Step 1· The approximate normality of concentrated HCl is 10 (say). A slightly greater than 0.1N HCl is prepared by dilution.

Step 2. Preparation of 0 1N primary base, i.e , sodium carbonate solution.

Step 3· Titration

Step 4. Calculation

Step 5: Dilution to get exactly 0.1N HCl.

Method: Pipette out about 3 ml of concentrated HCl and transfer to a 250 ml standard flask containing 100 ml of distilled water Mix Then add water upto the mark Stopper, invert and swirl several times for uniform mixing Lable as approximately 0 1N HCl solution

Weight accurately 0.530 gms of sodium carbonate crystals. Transfer to a 100 ml flask the weighed crystals through funnel with a jet of water. Dissolve the crystals completely. Add water upto 100 ml

mark. Stopper, invert and swirl well. Lable as 0.1N sodium carbonate solution

Take a clean burette. Rinse with 0.1N sodium carbonate solution and fill the solution in the burette. Remove air bubbles Then fill the solution exactly to zero mark Take a clean 20 ml pipet and rinse with the HCl solution. Transfer accurately 20 ml of the acid into a clean conical flask Add 3-4 drops of methyl orange indicator. Now run the base from burette into the flask carefully till the red color changes to pale orange color Note the burette reading Repeat the experiment for concordant values

Observations and calculations. Approximate normality of concentrated HCl is 10.

to obtain 0.1N, dilute 1 in 100

i e , dilute 1 ml HCl to 100 ml with water

To obtain slightly greater than 0.1 N, dilute about 3 ml HCl to 250 ml with water

Mol wt. of sodium carbonate = 106

$$\text{Equivalent weight of sodium carbonate} = \frac{106}{2} = 53$$

$$\text{Weight for 100 ml, 0 1N} = E \times S \times V \text{ gms}$$
$$= \frac{53 \times 0\ 1 \times 100}{1000}$$
$$= 0\ 53 \text{ gms}$$

Table VII-8. Burette reading

Trial No	*Initial burette reading*	*Final burette reading*	*Volume of base added*	*Volume of acid taken*
1	0	25 ml	25 ml	20 ml
2	0	25 ml	25 ml	20 ml

$$V_1N_1 = V_2N_2$$

$$20 \times N_1 = 25 \times 0.1$$

$$\therefore N_1 = \frac{25 \times 0.1}{20}$$

Normality of HCl = 0 125.

Now, use 0 125 N HCl and dilute to exactly 0.1N as follows.

$$V_1N_1 = V_2N_2$$

$$100 \times 0\ 1 = V_2 \times 0\ 125$$

$$V_2 = \frac{100 \times 0.1}{0.125} = 80 \text{ ml}$$

So pipet out exactly 80 ml of the HCl and dilute to 100 ml Lable the acid solution as 0.1N hydrochloric acid

Exercise Prepare 100 ml of 0 1N sulfuric acid. Using this prepare 100 ml each of 0 01N & 0.05N solutions.

2.3.8 3 Preparation of 0 1N sodium hydroxide

Because oxalic acid is a pure substance, it is possible to weigh out to prepare 0.1N solution.

Sodium hydroxide is not a pure substance, however it absorbs moisture and liquifies So an exact weight is impossible to make. Therefore, we prepare a 1N sodium hydroxide solution in a round about manner

A 1N sodium hydroxide is prepared through following steps.

Step 1: Preparation of slightly greater than 1N NaOH

Step 2: Preparation of primary standard oxalic acid, 1N.

Step 3 Determination of exact normality.

Step 4· Dilution to 1N

Preparation of slightly greater than 1N NaOH.

Sodium hydroxide is corrosive, handle carefully When pipetting do not get any in your month

The equivalent weight of NaOH is 40 To make a 1N solution we would need 3.0 gm for 220 ml. However, since we want a slightly greater than 1N solution we should use about 9 gm for 200 ml

Using balance weigh 9 gms of NaOH and prepare 200 ml solution Lable it as approximately 1N NaOH Store sodium hydroxide in a polythene bottle.

Preparation of 1N oxalix acid solution:

The equivalent weight of oxalic acid, analytical grade, is 63.033 To make a 1N solution 100 ml, weight 6 3033 g exactly and prepare 100 ml solution Lable it as 1N oxalic acid.

Titration: Pipet out 10 ml of 1N oxalic acid into a clean 250 ml conical flask Add 3-4 drops of phenolphthalein Take a clean burette, rinse with distilled water and NaOH solution Fill it to zero after making sure that no air bubble is in the nozzle of the burette. Clamp it to a stand vertically Now run NaOH carefully till the appearance of a faint pink color. Repeat the titration for concordant values Record the observations

Now, let the titration value be 9.4 ml Since the acid is 1N and the volume taken for titration is 10 ml , to obtain 1N solution dilute 94 ml. of NaOH to 100 ml

Label and store in a polythene bottle.

1N NaOH thus can be used as a secondary standard for the preparation of other acid solutions.

Determination of exact normality of sodium hydroxide supplied:

In order to determine the exact normality of slightly greater than 1N NaOH, the following two steps are necessary.

Step 1. Titration.

Step 2: Calculation.

Use 1N oxalic acid as primary standard. Using a 10 ml pipet place exactly 10 ml of 1N oxalic acid in 100 ml Erlenmeyer (conical) flask Add a few drops of phenolphthalein indicator (1% phenolphthalein in 95% alcohol) Fill the burette with NaOH, gently rotate the flask and add the NaOH drop by drop until the solution becomes faint pink color This is the end point Repeat the titration Tabulate your values.

Calculation.

Vol (volumme) of oxalic acid used = 10 ml (V_1)

Normality of oxalic acid = 1 N (N_1)

Vol. of sodium hydroxide used = V_2 ml (9.0 ml)

Normality of NaOH = N_2?

$V_1N_1 = V_2N_2$

$10 \times 1 = 9 \times N_2$

$$\therefore N_2 = \frac{10 \times 1}{9}$$

This gives the exact normality

Next if exactly 1N NaOH is required dilute 9 ml NaOH to 10 ml or 90 ml to 100 ml. Store in a polythene bottle and lable as 1N NaOH.

2 3 8 4 Preparation of 1N sulfuric acid.

Sulfuric acid is not a pure substance, consequently we must prepare 1N H_2SO_4 only by standardisation using the 1N sodium hydroxide prepared earlier. If not prepare exactly 1N Na_2CO_3 solution by weight.

1N H_2SO_4 is prepared by the following three steps

1 Preparation of slightly greater than 1N H_2SO_4
2 Determination of exact normality
3 Dilution to make 1N H_2SO_4

Sulfuric acid is very corrosive—handle very carefully. Do not pipet out the acid directly; use a graduated cylinder or rubber teat.

The equivalent weight of H_2SO_4 is $\frac{M}{2}$ i.e 49.039. To make a 1N solution we would need 9.80g for a 200 ml solution. However since we wish to make slightly stronger than 1N solution we should use more than 9.8 gm, say 12 to 13 gm for 200 ml.

1 ml acid weighs 1.84 gms (Sp. gr. 1.84). Therefore 13 gms measure

$$13 \times \frac{1\ 00}{1.84} = 7\ 0 \text{ ml}$$

Consequently 7 ml of H_2SO_4 is made upto 200 ml with water (add acid to water only) This forms a slightly greater than 1N H_2SO_4.

Prepare the solution in the manner given below Use AR or GR grade acid. The concentration of it is 95% (w/w).

Transfer 193 ml of distilled water into a clean 250 ml standard flask using a graduated cylinder. Carefully pipet 7.0 ml concentrated H_2SO_4 (AR grade) using a 10 ml serological pipet and rubber teat or use a graduated cylinder. Add carefully the acid to the flask Stopper, invert and swirl several times. Lable it as slightly greater than 1N H_2SO_4.

Next step is to determine the exact normality of the acid. Using a 10 ml pipet transfer 10 ml of slightly greater than 1N H_2SO_4 to a 250 ml Erlenmeyer flask. Add a few drops of phenolphthalein indicator. Fill the burette with 1N NaOH (or you prepare 1N Na_2CO_3 by weight and use methyl orange as indicator) Add NaOH to the acid drop by drop till the appearance of a faint pink color which remains permanent Note the burette reading and the volume of sodium hydroxide used. Calculate the normality of the acid as follows:

Volume of acid = 10 ml (V_1)

Normality	=	N_1(?)
Volume of base	=	10 9 ml (say V_2)
Normality of base	=	1N (N_2)
V_1N_1	=	V_2N_2
$10 \times N_1$	=	10.9×1
i e. N_1	=	$\frac{10.9 \times 1}{10} = 1.09$

Now dilute 1.09 N acid to get 1N as follows

Normality desired	=	1N (N_1)
Volume of acid desired	=	100 (V_1)
Normality of acid	=	1.09 (N_2)
Volume of acid to be taken	=	V_2(?)
.. V_1N_1	=	V_2N_2
100×1	=	$1.09 \times V_2$
.. V_2	=	$\frac{100 \times 1}{1\ 09}$
	=	91.74 ml

. Pipet out exactly 91 ,74 ml of acid and dilute to 100 ml. Store n a clean bottle and lable it as 1N sulfuric acid

2.3.9 Buffers

A buffer solution is employed in some of the reactions which have to be carried at a particular pH. This is possible because buffer solution resists pH changes upon addition of small portions of acid or base

A buffer system consists of two chemicals in solution one of which is a weak acid and the other Na or K salt of the same acid.

Eg: Acetate buffer is prepared by mixing sodium acetate and acetic acid Buffer solutions are used in various enzymic and other reactions There are a variety of buffer mixtures like phosphate buffer, citrate buffer, carbonate-bicarbonate buffer, etc

A buffer solution is labelled with the strength and the pH it maintains like, 0.2 M phosphate buffer, pH 7.8 A table of preparation of different buffer mixtures is given below:

2 3.9 1 Phosphate buffer

Solution a—M/15 KH_2PO_4 or M/15 NaH_2PO_4

Solution b—M/15 Na_2HPO_4

Mix solution a and solution b in the volumes given below (Table VII-9).

Table VII-9 Phosphate buffer mixture

pH	*mls of b*	*mls of a*	*pH*	*mls of b*	*mls of a*
5 8	8	92	7.1	66 6	33 4
6 0	12.2	87 8	7 2	72 0	28 0
6 2	18 6	81 4	7 3	76 8	23.2
6 4	26.7	73 3	7 4	80 8	19 2
6 6	37 5	62 5	7 5	84 1	15 9
6.8	49 6	50.4	7.6	87 0	13 0
6 9	55 4	44.6	7 7	89 4	10.6
7 0	61 1	38 9	7 8	91 5	8 5

2 3.9.2 Acetate buffer

pK 4 76

Solution a—0 2 M acetic ccid

Solution b—0 2 M sodium acetate

Mix solution a with solution b in the volume given below and make up the final value to 1 litre with water (Table VII-10)

Table VII-10 Acetate buffer mixture

pH	*mls of a*	*mls of b*	*pH*	*mls of a*	*mls of b*
3.6	463	37	5 2	105	395
3.8	440	60	5.4	88	412
4 0	410	90	5 6	48	452
4 2	368	132			
4 4	305	195			
4.6	255	245			
4 8	200	300			
5.0	148	352			

2 3 9 3 Tris—HCl buffer

pK = 8 08.

Volume (ml) of 0.2 M HCl to be added to 250 ml of 0.2 M tris (hydroxymethyl) aminomethane diluted to 1 litre with water.

Table VII-11 Tris-HCl buffer mixture

pH	*ml*	*pH*	*ml*	*pH*	*ml*
7 2	221	8 0	134	8 8	41
7.4	207	8 2	110	9 0	25
7 6	192	8 4	83		
7 8	163	8 6	61		

Note:

1. pK is the pH around (±1 pK) which the buffer shows maximum buffering action Farther and farther away from pK buffer shows lesser and lesser buffering action.
2. The two stock solutions of desired Molarities are prepared and mixed in the same ratio as given above.
3. Preparation of other reagents are dealt with under relevant chapters

2.4 Instrumentation

2.4.1 Colorimetry (Photometry)

2.4.1.1 Introduction

Most methods for determining the chemical constituents of blood and other fluids are based upon measurements of light absorption, in which a color produced by a substance whose concentration is *unknown*, is compared with that produced by a *known* amount of the same substance (called the *standard*), assuming in each instance that the intensity of colour is proportional to the amount of the colour-producing substance present. *Photometry* refers to the measurement of the light-transmitting power of a solution in order to determine the concentration of light-absorbing substance, present in it and the instrument used in the measurement is called a *Photometer* (or an *absorptiometer*). However, *photoelectric colorimeter* or simply, *colorimeter* has become common laboratory terminology.

2.4.1.2 Different types of instruments

Colorimeters and *spectrophotometers* measure the amount of light absorbed by coloured or colourless solutions, *turbidimeters* and *nephelometers* measure the light scattered by suspension, *fluorimeters* determine the fluorescence produced by absorbed light and *flame photometers* analyse the alkali and alkaline earth metal constituents by means of their emission spectra

2.4.1.3 Visual colorimetry and photoelectric photometry

Two general methods are used for measuring the colour intensities of unknown (called 'test') and 'standard' (1) The *visual* method, in this the colours are matched by the eye using a colorimeter of the *Duboscq type* (*visual colorimeter*) This is being replaced rapidly by (2) the *photoelectric photometry* method, in which the intensity of the color is measured by determining the amount of electrical current developed by a *photoelectric cell* when the light transmitted by the coloured solution falls upon it

2 4.1 4 Advantages of photoelectric instruments

Photoelectric instruments possess the following advantages over visual colorimeters:

(1) They employ light which is essentially monochromatic. Colorimetry and spectrophotometry are based on the laws of *Beer and Lambert*. The laws are true only with monochromatic light

(2) The "electric eye" (the photoelectric cell) is far more reliable and sensitive than the human eye.

(3) These instruments are more sensitive, and therefore permit the determination of smaller amounts of substances than visual colorimetry.

(4) A "reagent blank" is used which compensates for extraneous colour which may be present in a solution.

(5) Much greater speed and ease of colour measurement are the other virtues.

2 4 1.5 Beer-Lambert law

Photometry is based on two fundamental laws

(1) Beer's law and

(2) Lambert's law.

When light is absorbed its intensity is reduced. The amount of light absorbed is related to the concentration of the coloured compound and to the length of the solution traversed by the light beam These two statements are brief expressions of the above two laws, respectively That is, the amount of light absorbed depends on the number of absorbing molecules in its path. To get strict linearity between *absorbance* and concentration over a wide range one should use a monochromatic light, i e., as narrow a wavelength range as possible Law does not hold good for polychromatic light

2 4.1 6 Light, its component colours and their wavelengths

Day light or white light is a combination of a seven colours (i e., it is polychromatic). "VIBGYOR" is a code to remember the component colours in the order. The wavelength of light which the human eye can perceive ranges from 400 to 700 nanometers (nm*). Table VII-12 gives the wavelengths of component colours. Radiation of shorter wavelength, i.e , less than 400 is called *ultraviolet* (UV) light and radiation of longer wavelength, i.e., more than 700 is called *infrared* (IR) light

Table VII-12 Approximate wavelength of colours

Colour	*Wavelength (nm)**
Violet	400-435
Blue	435-500
Green	500-570
Yellow	570-600
Orange	600-630
Red	630-700

*nm is also called as millimicron (mμ)

1 nm = $\frac{1}{10^9}$ of a meter. Wavelength is also given in Angstrom (Å).

One nm = 10 Å. Thus the above wavelength range can also be written as 4000-7000 Å

Coloured solutions, after absorbing certain wavelengths of light transmit others Therefore, the colour of the solution depends upon the transmitted light. For example, a solution of hemoglobin appears to be red because it absorbs blue-green light and transmits the complementary colour—i e , red. To measure the concentration of a red solution light at about 540 nm is passed through the solution, and the amount of light absorbed is related directly to the concentration of the red coloured compound in solution. Selection of light of narrow wavelength is made with coloured glass *filters.*

Photoelectric colorimeters may be classified into two main categories· The first category comprises filter photometers and the second category comprises spectrophotometers which contain a diffraction grating or a prism instead of filters for the same purpose

2.4 1.7 Filters

Filters consist of selected glass (or sometimes dyed gelatin) which is capable of transmitting light over a limited portion of the spectrum only. The instrument is provided with a set of replaceable filters marked "V, B, G, Y or R" for violet, blue, green, yellow or red filters Instead of the above mark a number may be written on each which indicates the wavelength of light that filter transmits. For example, a filter of number '54' absorbs all light except that of wavelengths around 540 nm, which pass through. Other filters bear the following numbers: 42, 49, 59 and 65 In some type of instruments the filters are not removable and they are fixed on to a disc which can only be rotated to bring the appropriate filter in the light path

Filters are of limited specificity, and one that is designed to

transmit 540 nm may actually transmit light between 520 and 560 nm with a peak transmittance at 540 nm

2 4.1.8 Choice of light filter

This will usually be the filter which gives the highest *reading*—i.e., absorbs most light and which gives most nearly a direct proportionality in reading with different concentrations of coloured compound This may be determined by trial Although, there are occasional exceptions, it will be found that a filter of the complementary colour to the solution being explained is suitable; for example, a blue solution transmits the blue portion of spectrum and absorbs the red portion. Hence a red filter (Table VII-13) will transmit the light that is absorbed by the blue solution only. Red is complementary to blue In the case of red solution, blue light is absorbed, and therefore by using a blue filter greater sensitivity is obtained In subsequent exercises involving the use of photoelectric colorimeter, mention is made in each case of the appropriate filter to be used.

Table VII-13

Colour of the solution	*Colour of the filter*
Red to orange	Blue or blue-green
Green	Red
Blue	Red
Purple	Green
Yellow	Violet

2.4.1.9 Photocell (Photoelectric cell)

Photoelectric instruments supplant the human eye by a photocell These instruments may be classified into two main classes: (1) single-photocell and (2) double-photocell instruments Single-photocell instruments possess the advantages of simplicity of construction and of the fact that "readings" can be made rapidly on a direct-reading *meter* which does not require manual adjustment. This type requires an absolutely stable light source to eliminate the error due to a change in the light intensity during a reading The error due to variations in the light intensity is avoided by carefully balancing two photocells in the second type However, the simple single-photocell, light-filter type is generally cheaper and quite satisfactory for most purposes.

Photocell contains light-sensitive surface which releases electrons in numbers proportional to the intensity of the light impinging upon it A photocell (barrier layer cell, selenide cell) is the simplest of the detectors. More sensitive than photocell is the phototube.

2 4.1.10 Schematic diagram of a photoelectric colorimeter

Many photoelectric colorimeters are now on the market:
(1) Klett-Summerson Photoelectric colorimeter (double photocell)
(2) 'Photochem' photoelectric colorimeters (single-photocell).

This description gives the general principles common to most of the simpler models. The simplest type is illustrated in Fig. VII-4. It consists of a source of light (with a concave reflector)—usually a tungsten lamp—connected to constant voltage mains, an adjustable diaphragm for adjusting the intensity of light, a coloured glass filter, a cuvette of 1 cm internal diameter which contains the solution to be examined, a single-photocell to receive the radiation and a directly connected sensitive galvanometer

The monochromatic light is passed through an absorbing column of coloured solution taken in the cuvette From the absorbing solution, the transmitted light is directed upon the photocell which converts the radiant energy into electrical energy. The current produced under these conditions is read from the galvanometer The degree of deflection of the galvanometer needle depends on the amount of current generated, and this depends on the amount of light falling on photocell

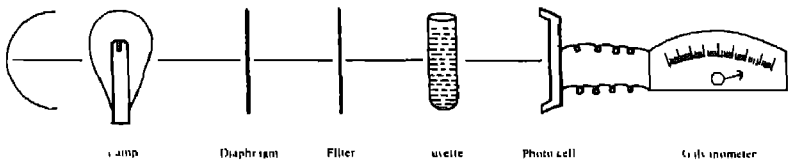


Fig VII 4 DIAGRAMATIC OUTLINE OF A COLORIMETER

2.4.1 11 Galvanometer

The magnitude of the current generated by the photocell may be measured in a meter, e.g. a galvanometer There are two types of scales marked in the galvanometer: (1) percent transmittance (%T) scale which is linear ranging from 0 to 100 and runs from left to right;

1 Switch-'on off
2 Dial
3 Knob
4 Deflection needle
5 6 Zero adjustment-knobs
7 10 Cuvet
8 Filter-carrier
9 Filter

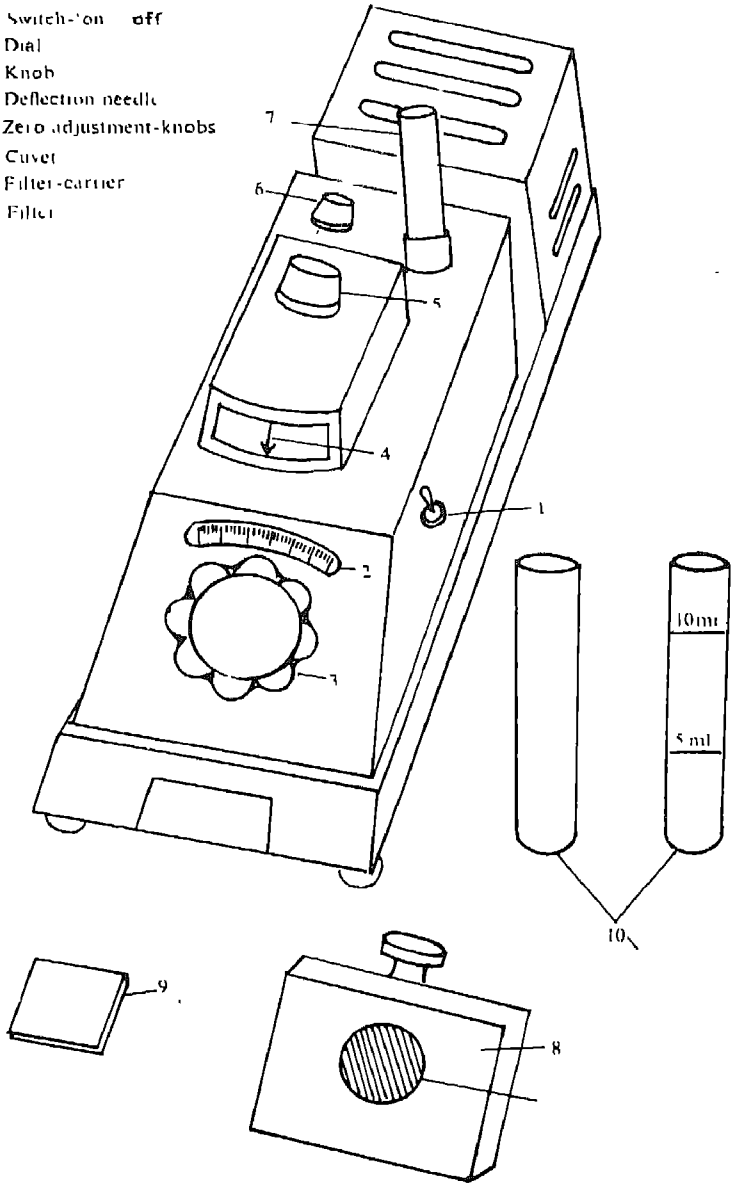
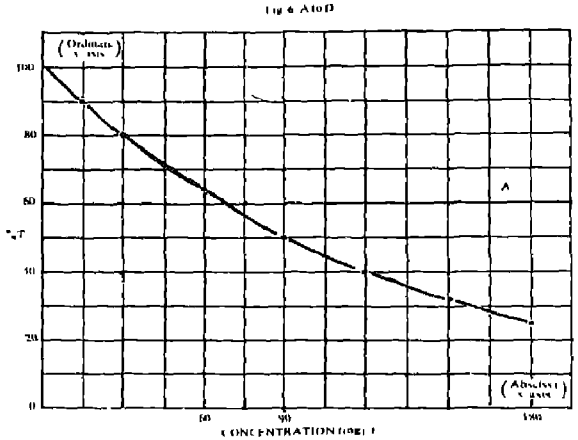
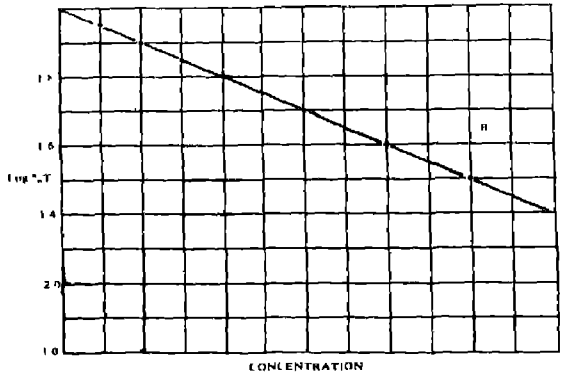
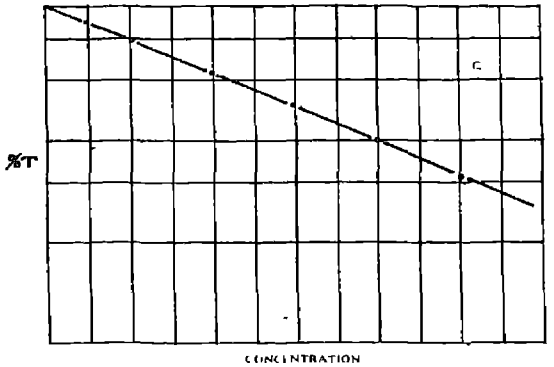


Fig VII 5 COLORIMETER -KLETT-SUMMERSON


A
CONCENTRATION


B
CONCENTRATION



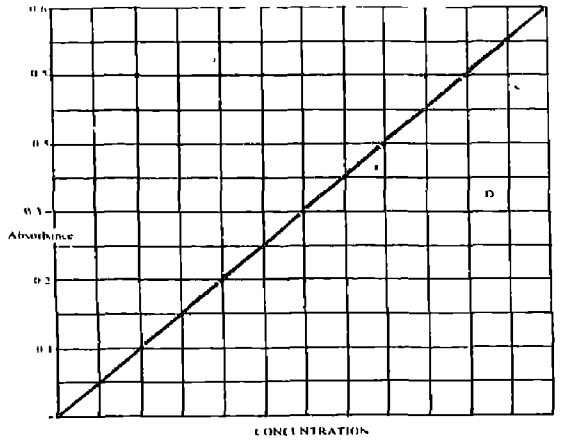


FIG VII A PHOTOMETRIC RELATIONSHIPS

(2) absorbance (also called optical density, O D or extinction, E) scale which is logarithmic ranging from 0 to 00 and runs from right to left with 'zero' absorbance coinciding with 100% T

2.4 1 12 Percent transmittance and absorbance

When light of an appropriate wavelength strikes a cuvette that contains a coloured solution, some of the light is absorbed by the solution, the rest is transmitted through the solution to the photocell. The proportion of the light that reaches the photocell is known as per cent transmittance (%T) and is represented by the equation.

$$\frac{I_t}{I_o} \times 100 = \%T$$

where I_o is the intensity of light striking the solution (incident light) and I_t is the intensity of the light transmitted through the solution The relationship between concentration of the solution and %T is not linear (See Fig. VII-6A).

The term "absorbance" (A) represents the negative logarithm of %T (i e., $-\log \frac{I_t}{I}$), absorbance increases directly with concentration of the substance (see Fig. VII-6D)

Absorbance is a measure of the amount of colour present in the solution. The greater the number of molecules of the coloured substance present, the greater is the absorbance. Or, in other words, the more the colour, the greater is the deflection of the galvanometer from its original (blank) setting. Thus, the concentration of the coloured substance present in a solution can be sensitively and accurately measured with a galvanometer.

The relationship of absorbance to concentration is expressed in the equation according to Beer-Lambert law (often referred to as Beer's law):

$$A = abc$$
$$= \log \frac{(100)}{\%T}$$
or $A = \log 100 - \log \%T$
i.e., $A = 2 - \log \%T$

where

A is the absorbance,,

a) is the absorptivity coefficient (a constant) of the coloured substance.

b) is the length of the light path through the solution, and

c) is the concentration of the coloured substance.

Since a linear relationship exists between absorbance and concentration but not between %T and concentration, absorbance is read out from 'absorbance scale' (optical density scale)

In some galvanometers only %T scale is available. In such cases absorbance can be calculated from the %T readings using the above mathematical relationship. Table VII-14 gives a few %T readings and their corresponding absorbances

Table VII-14 The relationship between light absorbed, transmittance & absorbance

% Light absorbed	*Transmittance (%T)*	$\frac{100}{\%T}$	*Absorbance* $\log \frac{100}{\%T}$
0	100	$\frac{100}{100}$	0
25	75	$\frac{100}{75}$	0.12
50	50	$\frac{100}{50}$	0.30
75	25	$\frac{100}{25}$	0 60
90	10	$\frac{100}{10}$	1.00
95	5	$\frac{100}{5}$	1 30
99	1	$\frac{100}{1}$	2.00
99.9	0 1	$\frac{100}{0\ 1}$	3 00
100	0	$\frac{100}{0}$	00

2 4.1 13 Different ways of plotting concentration of a substance & galvanometer readings

There are four different ways in which the relationship between the concentration of a substance and the galvanometer readings can be represented (Fig VII-6A to D).

In graph A, %T versus concentration, the resulting plot is a curved line. This type of relationship is difficult to use routinely because it makes interpolation and extrapolation difficult and inaccurate

Graph B, log %T vs concentration, results in a straight line with a negative slope (down from left to right).

Graph C, log %T vs concentration, results in a straight line with a negative slope on a special graph paper—'Semi-log'. Ordinate (y-axis) is plotted logarithmically and the abscissa (x-axis) linearly.

Graph D is the easiest relationship to use. It is a plot of absorbance vs concentration. This plot results in a straight line with a positive slope (up from left to right) That is, an increase in concentration results in an increase in absorbance. Each of these relationships or graphs is an expression of Beer's law

2.4.1.14 Instructions on the use of a photoelectric colorimeter

1 Choose the light filter (in a manner to be described later, see exercise No 1) and insert it in the filter slot in the instrument.

2. Wipe clean and properly insert the cuvette containing a sufficient volume of the 'reagent blank' (also called 'blank') solution in the cuvette holder Check for the correct placement of the cuvette Cuvette has an etched mark on it that has to be aligned with a mark on the cuvette holder.

   The primary purpose of the 'blank' is to read out any absorbance due to the reagent. The 'blank' should be prepared in the same fashion and with the same reagents as the specimens to be analyzed

3 Turn 'on' the instrument and allow it to 'warm up' for 5-10 minutes This brings the instrument to a "steady-state"—i.e. all parts of the electrical circuit come to equilibrium

4. Hold the switch pressed and turn the 'coarse' and 'fine control' connected to the diaphragm to vary the amount of light passing through the system until a full scale deflection of the galvanome-

ter is obtained. (Reading '0' on the absorbance scale or 100 on the %T scale) Release the switch*.

5 Insert the cuvette containing the 'standard' solution, press the switch and without disturbing the previous adjustment, read the absorbance Let it be 'S'. Release the switch.

6. Substitute the 'test' solution for the 'standard', press the switch and as before, read the absorbance. Let it be 'T'. Release the switch

7. After each reading check the instrument to see that the reading of the 'blank' has not changed from the initial setting of '0'. If there is a change repeat the operations '4 to 6'.

8 Switch off the instrument Empty the cuvettes, wash them thoroughly with tap water, rinse with distilled water and place them in their container.

9 Substitute the absorbance of 'standard' and 'test' in the following equation to calculate the concentration of unknown in the given sample of blood, urine, etc.

* Alternately, distilled water can be taken in the cuvette instead of 'blank' and the 'coarse' and 'fine control' are turned to get '0' absorbance in the galvanometer

Next, take 'blank' in the cuvette and read its absorbance Let it be 'B' Substitute 'standard' and then 'test' for the 'blank' and obtain the absorbances. Let them be 'S' and 'T' Subtract the absorbance of 'blank' from those of standard and test to get the true absorbances and calculate the concentration of unknown from the equation

$$\text{Concentration of unknown in mg/100 ml} = \frac{(T-B)}{(S-B)} \times \frac{\text{Concentration of Std.}}{\text{Volume of test}} \times 100$$

$$\text{Concentration of unknown in mg/100 ml} = \frac{\text{Absorbance of test}}{\text{Absorbance of Std.}} \times \frac{\text{Concen. of Std.}}{\text{Vol. of test}} \times 100$$

$$= \frac{T}{S} \times \frac{\text{Concen. of Std.}}{\text{Vol. of test}} \times 100$$

2 4 1.15 Observe these rules when using a colorimeter

1. Make sure that a filter is inserted into the filter slot. Failure to insert the filter may result in irreparable damage to the galvanometer

2. The cuvette should be filled with sufficient volume (a specified minimum) of the solution (such as 'blank', 'standard' to 'test') This prescribed volume is just enough to cover completely the aperture in the cuvette wall through which light beam travels. If a smaller volume is used, the light beam will be deflected by the meniscus and the open area above it, causing serious errors

3 If cuvette has a mark on it then turn the cuvette till the mark is in line with the mark on the cuvette holder. This ensures that the same surface is exposed to the light path and minimises any variation due to unevenness in the surface of the cuvette If a mark is not there it may be made near the top with a diamond pencil.

4. Always wipe the cuvette dry and free from finger marks before placing it in the cuvette slot

5. Make certain that no air bubbles cling to the inner surface of the cuvette. Gently tapping the cuvette usually will get rid of air bubbles, but a stirring rod may be used if necessary

6 Satisfactory results are only obtained with absorbances ranging from about 0.1 to 0.7. If the absorbance is more than 0.7 then the coloured solution should be suitably diluted and absorbance read or determination should be carried out with diluted sample of the supplied blood, urine, etc

7 It is impossible to compare solutions that are cloudy (turbid). All solutions must be perfectly clear

8 Cuvettes may be cleaned with mild detergents, but the use of hot concentrated acids or alkalies or any other cleaning agent which might etch surfaces or leave scratches as with brushing, should be avoided. Other useful cleaning solutions are a 5% (v/v) HNO_3 or a 1·1 (v/v) mixture of 5% (v/v) HCl and ethanol or methanol After having been soaked overnight in one of these solutions, the cuvette should be washed thoroughly with tap water, then rinsed with distilled water and finally with alcohol or acetone and kept inverted to dry.

2.4.1.16 Spectrophotometers

Instruments that are used to measure transmission at various wavelengths continuously are called spectrophotometer. These differ

from the filter photometers which can be used at only a relatively few wavelengths. Another limitation of the filter photometer is the breadth of the wavelength band which must be employed

The components of spectrophotometers are basically the same as colorimeters (see figure VII-7). In spectrophotometer filters are replaced with a continuously adjustable monochromator, capable of isolating a much narrower band of wavelengths. A monochromator is an assembly consisting of a dispersing element (a quartz prism, or diffraction grating) together with two narrow slits—an entrance and an exit—which control the spectral width The diffraction grating (a highly polished surface with numerous lines ruled on it) or the prism breaks white light falling on it into the spectrum of its component colours By adjustment only one particular portion can be allowed to pass through the opening.

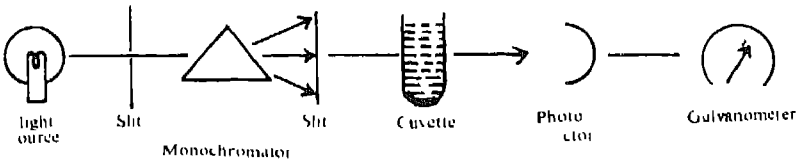


Fig. VII-7 COMPONENTS OF A SPECTRO PHOTOMETER

Spectrophotometers can be classified for convenience into three groups according to the spectral region over which they operate·

1. **Those with glass optics, sensitive roughly from 350 to 800 mm; Tungsten lamp is the light source.**
2. **Those with optical parts of quartz which extend through the** ultraviolet and visible, roughly 200 to 1000 nm, glass cuvettes cannot be used as glass absorbs at wavelengths less than 400 nm. Only quartz or plastic cuvettes that do not absorb UV radiation can be used for measurements. Either a hydrogen or deuterium lamp is the light source for the ultraviolet region.
3. **Infrared spectrophotometer covering the range from 1000 nm (1 u) upward.**

Spectrophotometers commonly used

1 Bausch and Lomb Spectronic 20 (visible range)
2 Systronics—spectrocolorimeter No 103 or 106 (visible range).
3 Bausch and Lomb Spectronic 21 (UV and visible range).
4. Beckman Model DB Spectrophotometer (").

2.4 1 17 Flame photometry (Flame emission spectrophotometry)

Flame photometer is a device for determining the concentration of metals in solution by measuring the intensities of the light emitted by them when their solutions are sprayed into a gass flame The basis of the measurement is that the colour of a burning flame is different for certain cations. The instrument offers one of the most convenient, accurate and precise measurements of Na^+, K^+ and others made in clinical laboratories The chemical procedures for Na and K are long and tedious, and the flame photometry forms a simple, accurate and speedy method determining their concentration in blood and other fluids with much less work than the chemical procedures require

Schematic diagram Figure VII-8 shows a schematic diagram of the basic parts of a flame photometer. The components consist of an aspirator, an atomiser, a flame, a monochromator, a phototube and a galvanometer. There are entrance and exist slits before and after the monochromator to narrow the beam of light.

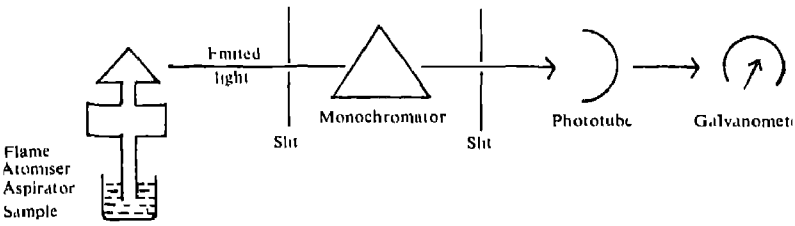


Fig VII-8 SCHEMATIC DIAGRAM OF A FLAME OF A FLAME PHOTOMETER

Principle· A standard solution of sodium or of potassium salt, or serum diluted with pure water, is sprayed by means of an atomiser as a fine mist into a special type of bunsen flame. This produces the yellow colour (with Na) or violet colour (with K), intensity of which varies with concentrations Emitted rays pass through the monochromator which excludes light from any substance other than that being

determined. filtered rays impinge on phototube and produce a current which deflects the galvanometer.

Note·
Determination using EEL Flame photometer is given in a later section

2.4.1 18 Measurement of the region of maximal absorption of coloured solution

Reagents·

1. 1% (v/v) HCl.
2 3% Cobalt acetate in 1% (v/v) HCl (A red solution).

Procedure:

1. Switch on the colorimeter and wait for 5-10 minutes
2. Place filter '43' in the filter slot
3 Take HCl ('blank') in the cuvette.
4. Adjust the galvanometer to read '0' absorbance.
5. Remove this cuvette and place the one containing cobalt acetate solution.
6 Record the absorbance.
7 Remove filter '43' and place filter '47' in the filter slot.
8. Remove the cuvette containing cobalt acetate solution and replace the one containing the 'blank'.
9 Repeat the above steps from '4'to '6'
10. Similarly record absorbances with their filters
11. Tabulate the readings as follows:

Table VII-15 Absorbances of some solutions with different filters

Filter	*Absorbance*
430 nm	0 32
470 nm	0 59
490 nm	**0.61**
550 nm	0 27
580 nm	0 10
610 nm	0 06
700 nm	0 03

From the above table it is seen that the absorption will be

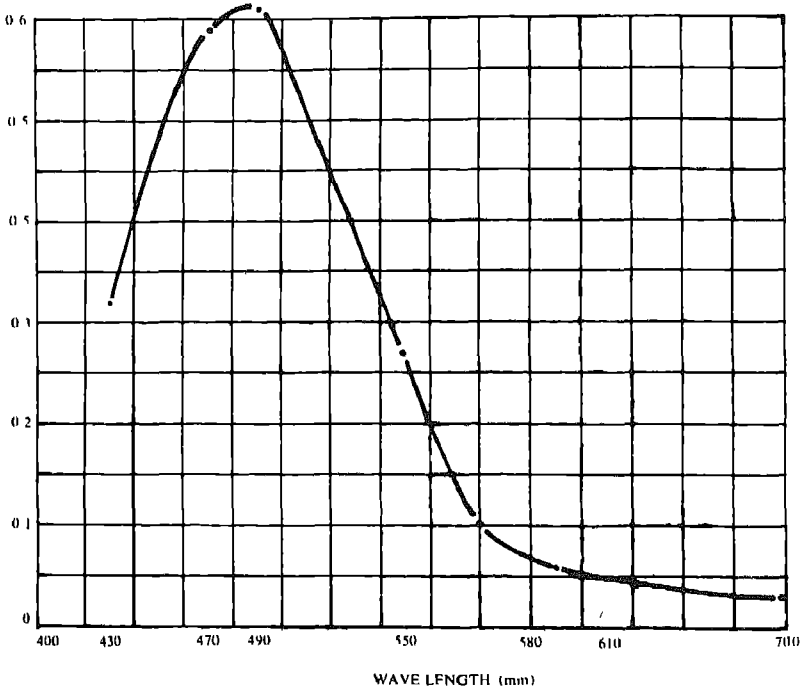


Fig VII-9 ABSORPTION SPECTRUM

maximum with blue filter (490 nm) indicating that for this solution, measurements should be made with this filter Also show the relationship on a graph (see figure VII-9) A graph showing the absorbance as a function of the wavelength is known as *absorption spectrum* (absorption curve) and because it depends on the chemical structure, a different spectrum is obtained with different structure. Absorption spectra have been likened to chemical "finger prints"

2.4.1.19 Verification of Beer's law

Reagents.

1. 1% (v/v) HCl.
2. 3% Cobalt acetate in 1% (v/v) HCl.

Procedure·

1. Arrange eight test tubes and mark them with a glass marking pencil from 1 to 8.

2. Pipette 6 ml of HCl into the first tube This tube serves as 'blank'
3 Pipette 0.5 ml, 1 ml, 2 ml, 3 ml, 4 ml, 5 ml and 6 ml of cobalt acetate solution into the remaining seven tubes.
4. Pipette 5.5 ml, 5 ml, 4 ml, 3 ml, 2 ml, 1 ml of HCl into tubes marked 2 to 7 to make up the total volume of 6 ml in each tube
5. Mix the contents carefully in each tube for uniform intensity.
6 Place filter '49' in the filter slot
7 Read absorbances of various tubes, setting the galvanometer to read '0' absorbance with 'blank' (first tube).
8 Tabulate the readings as in Table VII-16.

Table VII-16 Absorbanees are proportional to concentrations

Tube No	*Volume of cobalt acetate solution (ml)*	*Amount of cobalt acetate (mg)*	*Absorbance*	*%T*
1.	0	0	0	100
2.	0 5	15	0.05	90
3	1.0	30	0.1	80
4	2.0	60	0 2	64
5.	3.0	90	0 3	50
6	4 0	120	0 4	40
7.	5 0	150	0.5	32
8.	6 0	180	0.6	25

Plot the %T readings against concentration of the cobalt acetata in mg on a graph (see Fig. VII-6A). Note that the relationship is not linear. Similarly plot the corresponding absorbances against concentration of cobalt acetate in mg on a graph (see Fig VII-6D). A straight line is obtained up to a concentration of 180 mg cobalt acetate This verifies Beer's law, viz , absorbance increases linearly with concentration (in this case upto 180 mg)

From the calibration curve or standard curve thus obtained the concentration of "unknown" within this range can be deduced *

* But it must be recognized that the accuracy obtained in this waymay not be as great as that which will result from the simultaneous comparison of 'standard' with the 'test' solution

The reagents used in any procedure may vary slightly from day to day—e.g , by deterioration—and as a consequence the colour produced on one occasion may not be absolutely identical with that produced on another

2.4.2 Paper Chromatography

Chromatography is a technique used to separate solutes on the basis of their differential distributions between a two-phase solvent system where one phase (*mobile phase*) is caused to move over the other phase (*the immobile or stationary phase*) There are three types of chromatography, namely, partition, ion-exchange and absorption chromatography.

The chromatographic separations in practice may take one of the several forms· *column* chromatography in which the stationary phase is packed into glass columns, *thin-layer* chromatography in which the stationary phase is thinly coated onto glass plates and *paper* chromatography in which the stationary phase is supported by the cellulose fibres of a filter paper sheet

Paper chromatography is a *partition* chromatographic technique which has found widespread use Over the cellulose supporting medium the solvents flow. Water is considered as a stationary phase, as it is bound to the polar cellulose and the organic solvent that moves over the hydrated cellulose fibres is the mobile phase

Paper development· There are two techniques which may be employed for the development of paper *chromatogram:* (1) *ascending* and (2) *descending* method. The sample spots should be in a position just above the surface of the solvent so that, as the solvent moves vertically up the paper by capillary action, separation of the sample is achieved.

In the descending method the solvent moves downward under gravity Although ascending chromatography is often preferred because of the simplicity of the set up (see Fig. VII-10), the flow of solvent is faster in the descending technique

As the solvent passes through an area of the paper containing a solute, the solute will begin to partion itself between the aqueous and the organic phases in proportion to its relative solubilities in the two phases The more soluble the solute is in the organic phase, the faster the solute will be carried along by the organic phase. Conversely, the greater the affinity for water, the slower the solute will move with respect to the *solvent front.* Thus, if several compounds possess different solubility characteristics, theoretically each will progress across the paper at a specific rate which is different from that of any of the other compounds. The distance the solute moves in relation to the distance the solvent moves serves as a convenient means for

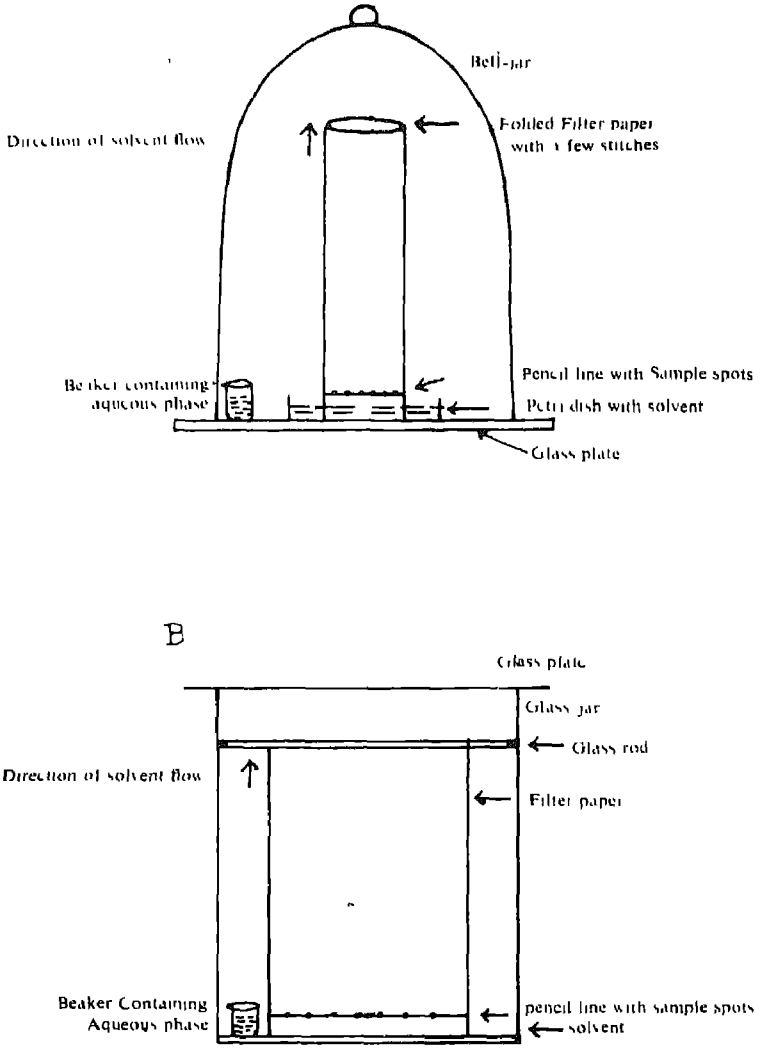


Fig VII 10 APPARATUS FOR ASCENDING CHROMATOGRAPHY (CROSS SECTION)

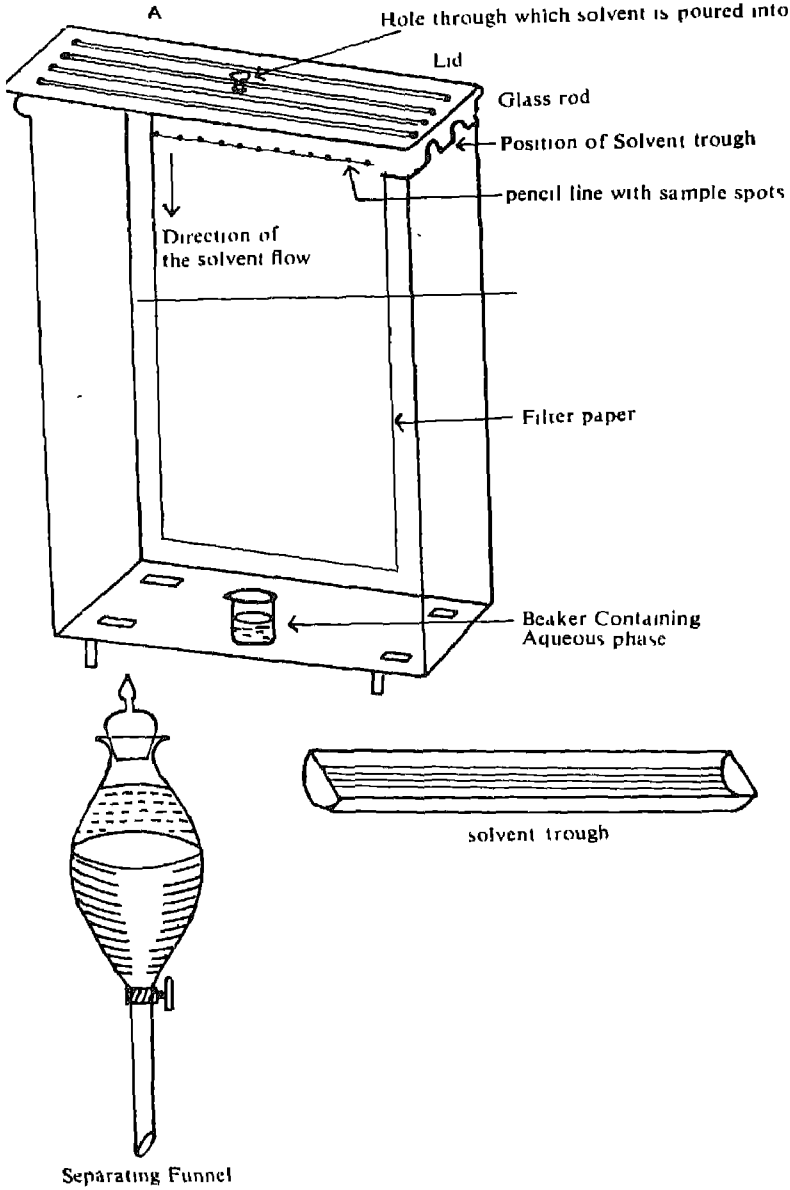


Fig VII-11 DESCENDING CHROMATOGRAPHY CHAMBER

identifying the solute. This *relative rate of flow* is the R_f value for the compound under the specified conditions of the experiment

$$R_f = \frac{\text{distance travelled by solute (measured to centre of the spot)}}{\text{Distance travelled by solvent}}$$

Several compounds may have the same R_f value in a particular solvent system, these can be separated by running more than one chromatogram each with a different solvent system It is always necessary to include *known compounds* in a chromatogram for comparative purposes.

Paper chromatographic separation of a mixture of amino acids

Apparatus. A chromatography chamber to demonstrate the descending type of chromatography

Solvent system:

The solvent is n-butanol acetic acid. water in the ratio 4·1:5 (v/v).

1. Pour 80 ml of n-butanol into a separating funnel (see figure VII-11) followed by 20 ml of glacial acetic acid and 100 ml of distilled water
2. Apply the stopper and with stopper and stop-cock held securely, mix the contents.
3. Open the stopper to release the pressure developed as a result of mixing.
4. Stopper again and mix. As before release the pressure.
5. Allow the layers to settle There will be two layers of solvent formed The bottom layer (about 100 ml)is the aqueous phase. Open the stop-cock and collect this alone in a beaker and keep it inside the chamber. This is the stationary phase. This is to saturate the chamber The upper layer is the mobile phase (the organic phase—about 100 ml)—the developing solvent

Filter paper This is a special quality filter paper. Whatman No. 1 is one such grade. The dimensions are 56 cm × 1 cm. Avoid direct handling of this paper.

Amino acid mixture given for identification:

Standard (known) amino acid solutions:

0.5 mg/ml solutions of phenylalanine, histidine, arginine and glutamic acid.

Staining reagent.

0 5% ninhydrin in n-butanol containing 10% water

Procedure

1. With a pencil draw a line along the 8 cm from one end. Starting 2 cm from the end, mark 5 positions on the line about 2 cm apart (see Fig VII-12A) Under each point write the name of the amino acid to be applied.
2. Apply 10 μl (microlitres) with a micropipet, of phenyl-alanine solution to the first marked position The spot should not be larger than about 0.5 cm in diameter. (This skill can be attained with a little practice). Allow the spot to dry before there is any need of apply the same solution to the same spot Amount of phenylalanine deposited in the spot in 5 μg*.
3. Wash the micropipet several times with the distilled water and now apply similarly 10 μl portions of other amino acid solutions and mixture taking care to wash the micro-pipet before the next solutions is applied
4. Fold the paper along a line 5 cm from the edge. Transfer the paper to the solvent trough in the chromatography chamber and anchor it with the help of a thick glass ro in the trough Make sure that the line with the applications is above the surface of the solvent that will be poured into the trough. Allow the paper to pass over the antisiphon rod and hang free Close the chamber to saturate with respect to the stationary phase kept in a beaker previously.
5. After equilibration for about 30 minutes fill the trough nearly half to three fourths with the developing solvent. Allow the solvent to run over the paper for 16-20 hours
6. When the solvent front has nearly moved to the other end of the paper, remove the paper carefully from the chamber, mark the

**Note*· Concentration of supplied solution is 0.5 mg/ml, i.e., 500μg (microgram)/ml, i.e., 500 μg/1000 μl; i e , 5 μg/10 μl.

solvent front with a pencil immediately and dry the paper in the air by hanging it to the clips.

Colour development of the chromatogram:

Since the amino acids are colourless they will appear as coloured spots only when the paper is sprayed with the staining reagent.

1 Spray the paper with the staining reagent using a spray gun (or a sprayer or even with a pipette if care is taken) Air dry for about 10-15 minutes and,

2 Then in an oven maintained at 150°C for 5 minutes. The amino acids appear as distinct purple spots (Fig. VII-12 B)

3. Circle the spots with pencil and mark the centres of such coloured spots

4 Measure the distances of these centres from the pencil line (i.e., the line of application of amino acids) and also the distance of solvent front from the same pencil line. Calculate the R_f values of each amino acid in the given mixture and identify them by comparing with the R_f values of the known amino acids (Table VII-17).

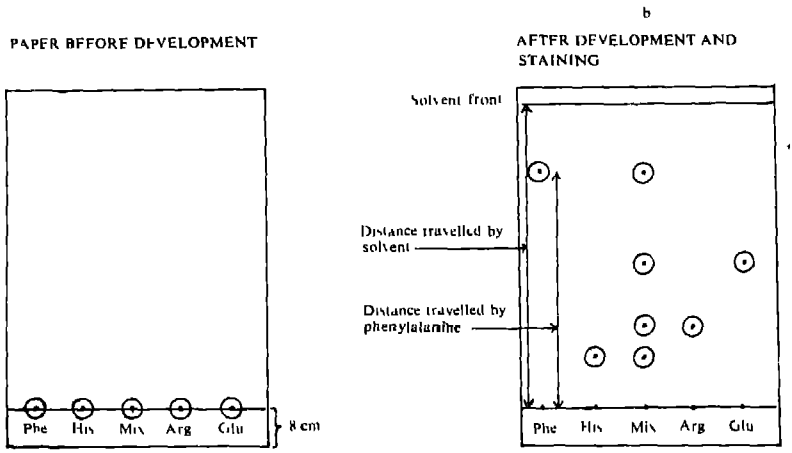


Fig VII-12

Table VII-17

Amino acid	R_f
Histidine	0 17
Arginine	0 19
Glutamic acid	0.29
Phenylalanine	0 65

2.4.3 Electrophoresis

Electrophoresis is the separation of the charged constituents of a solution by means of an electric current. A charged particle placed in an electrical field migrates towards the anode or the cathode depending upon the net charge carried by the particle. Molecules which have a similar charge will have different charge/mass ratios when they have differences in molecular weight. These differences form a sufficient basis for a differential migration

Electrophoresis is a valuable diagnostic tool in clinical biochemistry laboratories and is most commonly used for the separation of proteins found in serum. It is also useful in the separation of different forms of hemoglobins, immunoglobulins and isoenzymes

Agar, filter paper, cellulose acetate or agarose is used as *support medium.* They have large pores. At a pH of 8 6 all of the serum proteins carry a net negative charge and tend to migrate towards the anode (Gammaglobulins may stay at the point of application of

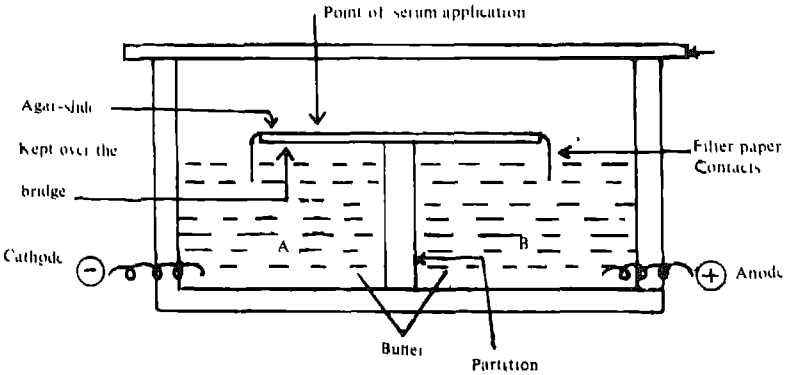


Fig VII 13 A CROSS SECTION OF ELECTRO PHORESIS CHAMBER

serum or move a little towards the cathode) Albumin carries a largest charge and therefore, moves the fastest, the gammaglobulins have the smallest net charge Serum proteins separate into five bands on cellulose acetate, etc The five bands starting from anode are designated as albumin, α_1-globulin, α_2-globulin, β-globulin and γ-globulin. Six bands are formed on agarose (the β-globulin splitting to give β_1-and β_2-)

The bands can be quantitated. However, this is not routinely done

Electrophoretic separation of serum proteins on agar gel equipment:

1 An electrophoresis chamber:

As shown in Fig. VII-13, an electrophoresis chamber consists of two compartments separated from each other by a dividing wall; one side contains the anode, and the other the cathode (platinum wires). Each side is filled to the same level with a buffer. A "bridge" across the top of the dividing wall holds the support material so that each end is in contact with the buffer in one of the compartments. Contact is made with filter paper strips.

2 Power pack (Power supply)·

This provides an output of about 120-150 volts with about 2 to 5 mAmp (milli amperes) of current being drawn per side. When a voltage is applied, current is carried across the porous medium from the cathode (negative pole) to the anode (positive pole) by the buffer ions.

Reagents:

1. Buffer

Barbital bfufer, pH 8.6, ionic strength 0.075. Dissolve 15.4 gm of sodium diethyl barbiturate (sodium barbitone) and 2 76 gm of barbituric acid in distilled water and make up to litre.

2. Staining solution:

A 1% solution of amidoschwartz 10 B (amidoblack 10B) in methanol containing 10% glacial acetic acid.

3. Wash solution:

3% acetic acid (v/v)

Principle: Thin filter paper strip soaked in serum is applied onto the agar support medium and a voltage is applied. After a definite period of 'run', the agar slide is removed The serum proteins would have separated into five distinct bands. Since they are colourless they have to be stained with the dye

The intensities of the coloured bands when compared with a normal pattern give an idea about the nature and relative concentrations of the resolved protein fractions

Procedure·

1. Fill the chamber with buffer, and level the liquid.
2. Dissolve 10 mg agar in 10 ml of warm buffer in a test tube (The support medium should be made in buffer to conduct the current) Deliver from a broad-tipped pipette 1.3 ml of this molten agar solution on a clean glass slide carefully without any air bubbles Allow the gel to set uniformly for a few minutes.
3. Keep this 'agar-slide' on the bridge in the chamber. Place two filter paper strips having the same width as the slide on both ends of the slide and connect to the two buffer compartments.
4 Soak a thin strip of filter paper (Whatman 3 MM, 1× 5 mm) with the serum sample and place it carefully on the agar gel at about one third distance from the cathode end of the slide.
5. Close the chamber, switch on the current and adjust the flow to 3 mAmp/slide
6. At the end of the two hours' run, switch off the current. Remove the agar slide and immerse in absolute alcohol for 30 minutes in a refrigerator. This denatures the proteins.
7. Dry the slide by keeping it at a height above the hot plate or by exposing it to infrared lamp and immerse in the staining solution for 2 minutes
8. Remove the excess dye by gently agitating the slide in three rinses of 'wash solution'.
9 Dry the slide. Observe the various bands of the electrophoretogram and compare with a normal pattern

Normal values: The normal adult has a serum protein concentration

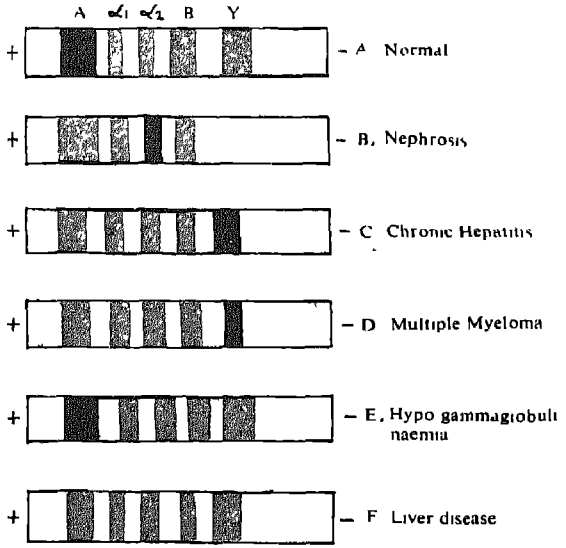


Fig VII-14 ELECTRO PHORETOGRAM (Normal and some typical cases) OF SERUM PROTEINS

that varies from 6.0 to 8.2 gm% Upon separation by electrophoresis, the following concentration in gm% and percentages are usually found·

Table VII-18 Serum protein concentration

Fraction	*Concentration*	*Per cent of total*
Albumin	3 5-5 2	*56*
α_1-globulin	0.1-0 4	*3*
α_2-globulin	0 5-1.0	*13 5*
β-globulin	0 6-1.2	*15 5*
γ-globulin	0 6-1 6	*12*

The electrophoretogram of serum proteins is helpful in detecting changes in the individual protein fractions and in detecting abnormal bands in certain diseased conditions (see Fig. VII-14).

The pattern in nephrosis reveals a low level of albumin and a marked rise of α_2-globulin. A small increase in β-globulin can also be seen in this condition (Figure VII-14B).

In chronic infection (hepatitis) a relative decrease in albumin

with a notable elevation in γ-globulin is observed

In multiple myeloma the characteristic feature is the presence of an abnormal band (M protein) usually between β-and γ-globulin bands, closer to the γ-band.

In hypogammaglobulinemia a considerable drop in γ-globulin is readily observed A slight increase in α_2-globulin is also seen; but is not a common finding in hypogammaglobulinemia

In chronic liver diseases a decrease in albumin band is observed Occasionally an increase in γ- and β-globulin is also seen (Figure F)

Quantitation of protein fractions·

This is done by mounting the agar-slide in an appropriate holder and passing through a recording *Densitometer* Wave-length of filter used is 520 nm This gives a densitometric scan of dyed electrophoretogram

In the absence of a densitometer, the intensity of dye in each band can be determined by cutting the bands individually from the dyed agar-slide and carefully transferring the scrapings to test tubes, eluting the dye from each band with 2 ml of 0 1 M NaOH, and then recording the absorbance at 520 nm. The absorbance of each band is read against a blank made by elution of an unstained portion of the agar-slide.

$$\text{\% total protein in a band} = \frac{\text{Absorbance of that band}}{\text{Sum of absorbances of all bands}} \times 100$$

PART III

DIAGNOSTIC TESTS ON URINE

3.1 Collection & Preservation of Urine Specimens

Collection

For most of the qualitative tests on urine a random specimen of urine is satisfactory But for quantitative determinations like urine phosphate, calcium, uric acid, ammonia, etc a 24 hour specimen is most suitable because the concentrations vary at different times of the day. So collection of 24 hour urine sample is imperative

The patient completely empties the bladder at 8 O'clock in the morning. This sample is discarded. Thereafter he collects in a 5 L bottle all the urine passed during the day and the subsequent night till the next morning 8 O'clock. The 8 O'clock morning sample of urine is also collected.

If the 8 O'clock sample on the day of starting collection is collected the next morning 8 O'clock sample is discarded. Thus a 24 hour urine specimen is procured

Changes on Keeping

1. Bacterial action Urea level is affected so also ammonia and pH.
2. When urine becomes alkaline phosphates precipitate
3. Uric acid crystallises out.

Preservatives for urine

The above changes have to be prevented So 50 ml of 2 M hydrochloric acid is used as a preservative for collection for estimations of urea, ammonia, total nitrogen and calcium. However uric acid precipitates out. Liquid paraffin, chloroform, toluene, petroleum (light), thymol and formalin are used as urine preservatives. A few millilitres of the one of these is put into a 5 L bottle and is given for collection These form a layer over urine and hence prevent entry

of bacteria into urine While transferring urine for estimations it is poured into a separating funnel and then transferred into a beaker from which it is pipetted out This is to avoid these liquids getting into the pipet

One of the above preservatives is satisfactory for almost all the routine investigations and estimations on urine.

3.2 Physical Characteristics of Urine

3.2.1 Normal Urine

It is desirable to review certain important features of the composition of normal urine, an understanding of which may aid in the interpretation of changes that occur in disease

Table VII-19 Physical characteristics of normal urine and alterations in diseases

Parameters	*Normal features*	*Remarks*
1 Volume	**Quantity excreted by** normal subjects ranges from 1000-2000 ml per day (average/1500 ml)	Volume is influenced by intake of fluid. Excessive perspiration and strenous exercise decrease the output Polyuria (an increased output)—occurs **in diabetes mellitus &** diabetes insipidus, after taking drugs such as digitalis, salicylates, etc. Oliguria (diminished excretion)—in nephritis, fevers, diarrhoea and vomiting Anuria (Total suppression of urine)—may occur for a period in shock, acute nephritis, incompatible blood transfusion, mercury poisoning and bilateral renal stone
2 Specific gravity	Ranges from 0 012-1 024	Specific gravity is directly proportional to the concentration of solutes excreted It varies inversely with the volume of urine excreted It is as low as 1.001 when water intake is high It is as high as 1.04 when vol. of urine is restricted It is increased **in acute nephritis, fevers and diabetes mellitus** It is decreased in diabetes insipidus.
3 pH	Ranges from 4 8-7.5 (average 6 0)	Acidity is mainly due to acid phosphates (NaH_2PO_4)

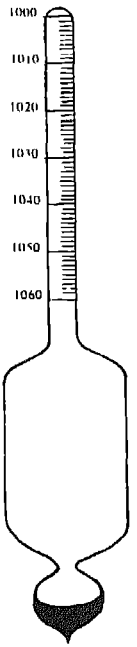
			High protein diet gives rise to a more acidic urine Urine is alkaline when the diet is rich in vegetables and fruits Significantly acidic urine in fever, diabetes and in starvation Significantly alkaline after alkali therapy and retention of urine
4	Titrable acidity	200-300 ml of N/10 NaOH	In starvation and diabetic ketoacidosis acidity of urine is increased Acidity is decreased in alkalosis.
5	Colour	Amber yellow (Straw colour) Perfectly clear and transparent when voided	A fresh sample of urine on standing develops a faint cloudiness due to the Presence of traces of nucleoprotein & epithelial cells Turbidity may also be due to precipitation of insoluble phosphates of Ca and Mg May depend upon the products excreted (and also on the drugs ingested) Smoky brown due to presence of blood Yellow due to presence of bilirubin Black due to presence of melalin Milky due to presence of pus, bacteria or epithelial cells and lipids
6	Odour	Described as 'aromatic'	When left standing it develops ammonical smell due to bacterial action on urea

3.2.2 Determination of Specific Gravity of Urine

1. Urinometer
2. Thermometer
3. Urine glass or measuring cylinder.

Urinometer (hydrometer) consists of a thin stem graduated from 1000 to 1060 corresponding to specific gravities of 1 0 to 1.06. **The bulb at the bottom is suitably weighted. Urinometer is graduated** at 60^0F *

$$60^0F^* = (60 - 32) \times \frac{5}{9} = 28 \times \frac{5}{9} \simeq 15^0C, \simeq = \text{approximate}$$



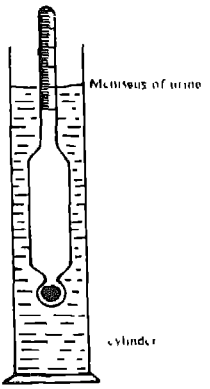


Fig VII 13 URINOMETER

Procedure:

1 Take sufficient fresh urine in the urine glass

2 Allow the urinometer to float in urine without touching the side Observe the reading corresponding to the meniscus of urine (observed **sp.gr.)**

3. Note the temperature of urine with a thermometer.

4 Apply correction for temperature as follows·

For every 3^0C rise over the temperature of calibration (15^0C) a correction factor of one is added to the observed specific gravity.

Example

Observed specific gravity is 1015.
Temperature of urine is 37^0C.
Calibration temperature of urinometer is 15^0C
Therefore, difference between these two = 22^0C

Dividing this by 3, $\frac{22}{3} \simeq 7$ (the factor to be added).

Therefore, true specific gravity = 1015 + 7 = 1022

Applying the decimal point, the specific gravity of the given urine after temperature correction = 1.022.

3.3 Normal Constituents of Urine

Table VII-20 Chemical characteristics of normal urine and alterations in diseases

Inorganic constituents	*Amount*	*Conditions*
1 Chloride	On an average diet 8-15 gm of chloride as NaCl are excreted per day	Depends on dietary intake. Depends on loss via sweat Excretion is more in Addison's disease in which there is a deficiency of adrenal cortical hormones Excretion is more in salt-losing nephritis Excretion is decreased in Cushing's syndrome (hyper secretion of adrenal cortical hormones) and in adrenal steroid therapy
2 Sulphate	About 1 gm of sulphur	About 85-95% of this is excreted as inorganic sulphate Rest as organic sulphate and as neutral sulphur
3 Phosphate	About 1 gm	Mostly as acid phosphate Basic phosphate is much less. Excretion is increased in bone diseases
4 Ammonia	About 0 5-0.8 gm	Excretion is increased when acid-forming foods are taken Decreased by ingestion of base-forming foods Decreased by renal failure and metabolic acidosis. Increased in diabetes mellitus (ketoacidosis)
5 Calcium	Around 200 mg	Increased in parathyroid disease

6	Urea	20-30 gms	End product of protein meta-bolism Excretion depends on protein intake This constitutes 80-90% of the total nitrogen excreted Decreased in renal disease (renal failure)
7	Uric acid	0 6-1 gm	End product of purine metabolism. Output is increased in Leukemia because of destruction of nuclear material Decreased in renal failure.
8	Creatinine	1-2 gm	Excretion is related to the amount of muscle mass Excretion remains constant in an individual For this reason its estimation helps to check the reliability of 24 hours urine collection Decreased in renal failure
9	Ethereal sulphate (organic sulphate) e.g indican	About 100 mg	Indican is a product of putrefaction Large amounts are excreted in 1 Cancer of the stomach 2 Peritonitis, and 3 Prolonged constipation
10	Urobilinogen	Traces (upto 4 mg/day)	This is formed from bilirubin in the intestine. Decreased in obstructive jaundice Increased in hemolytic jaundice *Note*· On standing **urobilinogen** is oxidised to urobilin Test for urobilinogen should be done with a fresh urine

Also present in normal urine are K^+, traces of Mg^{++} and bicarbonate Numerous other substances are of limited clinical importance or are present only in traces. They are hippuric acid, oxalic acid, amino acid, enzymes like amylase, hormones, vitamins like C, pigments, 5 hydroxyindole acetic acid, glucuronides, etc

'It is often desirable to collect and test qualitatively samples of urine at random However, it is necessary to analyze a sample taken from an entire 24 hours excretion if dependable data are required regarding the quantitative composition of the urine.

3.3.1 Qualitative Tests for Normal Constituents of Urine

Reagents

1. Concentrated HNO_3
2. 1% Acetic acid
3. 10% $BaCl_2$
4. 3% $AgNO_3$
5. 2% Potassium oxalate
6. Ammonium molybdate (solid)
7. Red litmus paper
8. 2% and 20% Na_2CO_3
9. 10% NaOH
10. Concentrated HCl
11. Saturated picric acid solution (a 1.2% solution)
12. Sodium hypobromite reagent 100 ml of 40% NaOH containing 10 ml liquid bromine.
13. Urease suspension· Grind 10 gm horse-gram powder (*Dolichos uniflorus*, a rich source of urease) with 100 ml of 30% ethanol
14. Ammoniacal $AgNO_3$. To 2.6 gm $AgNO_3$ in 100 ml water add enough NH_4OH to redissolve the precipitate which forms upon the first addition of NH_4OH.
15. Phosphotungstic acid reagent Transfer 100 gm sodium tungstate to 150 ml water. Add to this 16 3 ml orthophosphoric acid and 16 8 ml concentrated H_2SO_4. Mix the solution and boil gently for 2 hours using reflux condenser. Cool the solution and dilute to 1 litre.
16. Enrlich reagent. To 100 ml water in a brown bottle add 0.7 gram p-dimethyl amino benzaldehyde followed by 150 ml concentrated HCl Mix

The various tests are given in Table VII-21

Table VII-21 Various qualitative tests for normal constituents of urine.

Constituents	*Test detail*	*Observation*
1 Chloride	To 2 ml urine add 0 5 ml conc HNO_3 and 1 ml $AgNO_3$	White precipitate of AgCl *Note.* This changes to brown after sometime
2 Sulphate	To 2 ml urine add 2 ml $BaCl_2$	White precipitate of $BaSO_4$.
3 Phosphorus	To 5 ml urine add 5 drops of conc.	Canary yellow precipitate of

	HNO_3 and a little ammonium molybdate. Warm.	ammonium phospho molybdates
4 Calcium	To 5 ml urine add 5 drops of acetic acid and 5 ml potassium oxalate Use a glass rod to scratch the side of the test tube	A trace white precipitate of calcium oxalate
5 Ammonia	To 5 ml urine add 2% Na_2CO_3 till the solution is alkaline to litmus (red paper should turn blue). Boil the contents Hold a piece of moistened red litmus paper at the mouth of the test tube	Red litmus turns blue by the evolved ammonia
6 Urea	(a) *Sodium hypobromite test* To 2 ml urine add 5 drops of sodium hypobromite reagent.	Brisk effervescence of nitrogen
	(b) Urease test Lable 2 test tubes as 'test' and 'control' To both add 5 ml urine	
	To the 'test' add 2 ml urease suspension and to the 'control' 2 ml urease which has been heated (to inactivate the enzyme) Leave the tubes for 15 minutes at room temperature Add 2 drops of phenolphthalein to both	Pink color only in 'test' due to the increase in pH caused by the formation of ammonium carbonate (an alkaline compound)
7. Uric acid	*(a) Schiff's test* Wet a piece of filter paper with a few drops of ammoniacal $AgNO_3$ Add a few drops of urine on to the same portion	Formation of black colour (after sometime) due to the precipitation of silver
	(b) Phosphotungstic acid reduction test To 2 ml urine add a few drops of phosphotungstic acid reagent and a few drops of 20% Na_2CO_3	Blue colour due to reduction of phosphotungstic acid to 'tungsten blue'.
8 Creatinine	*Jaffe test* label 2 test tubes as 'test' and 'control' Take 2 ml urine in 'test' and 2 ml water in 'control', add to both 2 ml picric acid & few drops of NaOH.	Orange colour in 'test' due to the formation of creatinine picrate. However, the colour remains yellow in control.

9	Ethereal sulphate	To 5 ml urine add 2 ml $BaCl_2$ and 2 ml conc HCl Mix and filter Divide the filtrate into 2 portions Boil one and compare with the other ('control'—unboiled one)	A trace turbidity over that in the "control"
10	Urobilinogen	*Ehrlich's test* To 5 ml of freshly voided urine add urine add 1 ml Ehrlich reagent Mix Let stand for 5 minutes	A red colour when viewed through the mouth of the test tube (hold a white paper below the test tube)

3.3.2 Determination of Urea, Uric Acid, Creatinine, Calcium, Phosphate, Sodium, Potassium and Chloride

Estimation of urea in urine

Apparatus.

Doremus ureometer

Reagent

Sodium hypobromite reagent.

Sodium hypobromite reagent 100 ml of 40% NaOH containing, 10 ml liquid bromine.

Doremus ureometer consists of a narrow open limb graduated in ml. This limb is connected to a broader closed limb through a stopcock. The closed limb, which ends in a bulb, is graduated in gm/ml Each small division is equal to 0.001 gm.

Urine is treated with sodium hypobromite to form nitrogen.

$$H_2NCONH_2 + 3NaOBr + 2NaOH \rightarrow N_2 + Na_2CO_3 + 3NaBr + 3H_2O$$

From the amount of nitrogen liberated the quantity of urea is directly read.

Procedure.

With the apparatus held upright, pour sodium hypobromite reagent nearly enough to fill the bulb.

With the stopcock completely closed, tilt the apparatus to fill the closed limb completely with the reagent. Avoid air bubbles entering in.

Pour urine into the open limb to the 'O' mark.

Open the stopcock carefully to deliver one ml of urine into the apparatus. Nitrogen collects in the closed limb.

After frothing subsides, take the reading corresponding to the

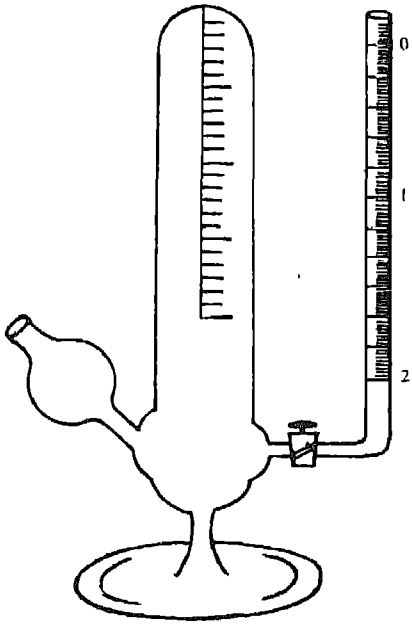


Fig VII 16 DOREMUS UREOMETER

meniscus of the reagent. Express the value of urea as gm/100 ml urine.

Significance

Daily excretion is 20-30 gm. Increased in febrile or wasting diseases. Measurement indicates the ability of the kidney to excrete

Determination of chloride in urine:

This determination is done in exactly the same way as serum chloride

Determination of calcium in urine:

This determination is done in exactly the same way as serum calcium

Determination of uric acid in urine:

This determination is done in exactly the same way as uric acid in serum but after diluting the urine 1 in 10

Determination of creatinine in urine:

This determination is done in exactly the same way as creatinine in serum but after diluting the urine 1 in 25.

Determination of urine phosphate:

This determination is done in exactly the same way as serum phosphate but after diluting urine 1 to 10.

Determination of urine sodium and potassium:

This determination is done in exactly the same way as serum sodium and potassium but after diluting the urine 1 in 100.

3.3.3 Determination of Titrable Acidity and Ammonia in Urine

Maintenance of intracellular pH in the tissues and in the body fluids within limits, is vital for normal cellular function. The kidneys by their ability to increase or decrease the secretion of H^+ plays an important role in H^+ homeostasis. The H^+ secreted by renal tubules is buffered in the tubular fluids by the phosphate system (Na_2HPO_4/NaH_2PO_4) and also by the ammonia synthesized in renal tubular cells (NH_3/NH_4^+). The H^+ secretion is always accompanied by reabsorption of Na^+ in the renal tubules.

The titrable acidity of urine is mainly due to acid phosphate and to a lesser extent due to weak organic acids. Titrable acidity can be determined by titrating urine with standard alkali with phenolphthalein as indicator.

$$NaH_2PO_4 + NaOH \rightarrow Na_2HPO_4 + H_2O$$

Since calcium interferes during titration due to its precipitation as calcium phosphate, it is removed completely as calcium oxalate before the estimation

Ammonia in urine arises from the hydrolysis of glutamine (60%) and due to the oxidative deamination of amino acids (40%) in the kidney.

Ammonia is estimated by the formol titration method When neutral formaldehyde is added to a solution containing ammonium salts, H^+ ions are liberated which can be titrated with standard alkali. Hexamethylene tetramine is the other product in this reaction

$$4\ NH_4^+ + 6\ HCHO \rightarrow 4H^+ + N_4(CH_2)_6 + 6\ H_2O$$

Reagents.

(1) 0.1 N NaOH
(2) Potassoium oxalate (solid)
(3) Phenolphthalein indicator (0.2% solution in ethanol)
(4) Neutralised formalin

Procedure·

(A) Titration for titrable acidity·

Pipette 25 ml urine into a 250 ml conical flask.
Add 2 spatula of potassium oxalate to precipitate the calcium.
Add 2 drops of phenolphthalein.
Rotate the flask for 1-2 minutes and titrate against NaOH.
Note the titre value (A ml) when a permanent pale pink colour appears.
Preserve the contents for 'ammonia' estimation.

(B) Titration for 'ammonia':

Note the initial reading of the burette
Add 5 ml of neutralized formalin to the above flask.
Pink colour disappears as a result of liberation of H^+. Titrate against NaOH until the pale pink colour reappears.
Record the titre value (B ml).
Repeat both the titrations till concordant values are obtained.
Tabulate the burette readings.

Table VII-22

Trial No	*Initial reading of the burette*	*Final reading of the burette*	*Volume of alkali required for*	
			Titrable acidity	*Ammonia*
1	0	4	4	—
	4	6	—	2

Calculation for acidity:

Volume of 0.1 N NaOH required to neutralise titrable acidity in 25 ml urine = A ml

Volume of alkali required for 100 ml urine = A × 4 ml

i.e , titrable acidity of 100 ml urine = 4 A ml 0 1 N NaOH
Assuming the output of urine for 24 hours to be 1500 ml,

titrable acidity of urine = **4 A × 15 ml/day.**

Calculation for ammonia:

Volume of aklaki required in this titration for 25 ml urine = B ml

Volume of alkali required for 100 ml urine = **B × 4 ml**

Since 1 ml of 0.1 N NaOH = **1.7 mg NH_3**

Ammonia content of 100 ml urine = **4 B × 1.7 mg**

or, ammonia content of urine = **4 B × 1.7 × 15 mg/day**

Clinical significance:

Titrable acidity in urine varies from 200-300 ml (20-30 millieq-uivalents) per day. The excretion of ammonia per day is in the range of 0 5-0.85 gm (30-50 milliequivalents).

Titrable acidity and ammonia in urine are generally low on carbohydrate-rich diet and high on high-protein diet

In starvation and in diabetic ketoacidosis the values are increased

Titrable acidity is decreased in alkalosis.

3.3.4. Urea Clearance Test

The kidneys remove urea from blood and is excreted in urine. The ability of kidneys to excrete urea is assessed by performing the urea clearance test.

Urea clearance is dofined as the mls of blood cleared of urea by the kidneys per minute. It is expressed as mls/minute.

Specimen·

1. 1 hour urine sample.
2 Venous blood.

Method.

1. Estimation of urea in urine by Doremus method
2. Estimation of urea in blood by Nesslerisation or diacetyl monoxime method.

Performance of the test:

Instruct the patient to avoid exercise before the test. It interferes with the result.

The test is done in the morning. One hour after breakfast ask the

patient to empty the bladder completely Discard the urine Note the time Give him a glass of water to drink Exactly one hour after ask the patient to empty the bladder. Collect the sample Label it as 1 hour sample. Measure the volume Collect venous blood for urea estimation Exactly after 2 hour ask the patient to empty the bladder completely again in a second container. Measure the volume of 2 hour sample The average excretion of urine per minute is calculated.

Eg 1 hour volume say is 120 ml, 2 hour volume is say 168 ml

$$\text{urine excreted per minute} = \frac{120 + 168}{60 + 60}$$

$$= \frac{288}{120} = 2\,4$$

$$= 2.4 \text{ ml/minute}$$

Now estimate urea in urine and Blood samples

Calculation·

Urine urea is mgs/100 ml = U

Blood urea in mgs/100 ml = B

Urine excreted per min = V

$$\text{Urea clearance} = \frac{UV}{B} \text{ mls/minute.}$$

The formula $\frac{UV}{B}$ for urea clearance holds good, when urine excreted per minute is $\geq$ 2 ml per minute.

If urine excreted per minute is less than 2.0 ml,

$$\text{Urea clearance} = \frac{U\sqrt{V}}{B}$$

Normal clearance is between 60-95 ml/min with an average of 75 ml/min Diminished clearance is found in renal impairment.

Note

It is better to collect 24 h urine sample.

3.3.5. Creatinine Clearance

The test is performed in exactly the same way as urea clearance test.

Serun and urine creatinine in mgs/100 ml are estimated by Jaffe's method dealt else where

Creatinine clearance averages to 120 ml/min with a range of 95-140 ml/min for men and 85-125 ml/min for women. Clearance is diminished in renal impairment.

3.4. Abnormal Constituents of Urine

A Many of the substances considered here as pathological constituents of urine are present in small amounts in normal urine. Hence their pathological significance may be more a question of the amount present than of their actual presence or absence from urine It is generally true, however, that the usual qualitative tests for these substances are of such sensitivity as to yield an essentially negative result when applied to normal urine, but to respond readily when unusual amounts are present. In this connection it is well to remember that a single specimen of urine may be sufficiently concentrated or otherwise influenced by dietary or other factors as to yield a positive result with a particular test, the significance of which disappears, however, when considered in the light of the total 24 hour excretion Whenever possible, it is recommended that urine tests be carried out on a portion of the well-mixed and properly preserved 24 hour urine before interpreting results; if this is not feasible, the overnight sample collected early morning should be used.

In disease, the urine analysis may show reducing sugar, albumin, pus, blood or bile These may indicate a disease of the kidneys or point to disorders in other parts of the body. The analysis of the urine thus becomes a substantial spoke in the wheel of diagnosis an the physician turns to the technician for information regarding its deviation from normal values.

Table VII-23 Chemical Constituents

Constituent	*Condition*
1 Albumin	*Normal urine may contain a trace* *Albuminuria is the presence of detectable amount of Albumin* *Proteinuria is a general term* *Characteristic of kidney disease—nephritis and nephrotic syndrome* *False albuminuria found in some due to erect posture*
2 Glucose	*Normal urine may contain a trace*

		Glycosuria is the presence of a detectable amount of glucose *Occurs in diabetes mellitus, renal diabetes, emotional disturbances, endocrine disorders.*
3.	Ketone bodies (acetone, acetoacetic acid, β-hydroxy butyric acid)	*When glucose is not oxidized, the body endeavours to compensate by oxidising fats.* *Excreted in diabetes mellitus and starvation* *Their presence indicates acidosis, a decrease in the alkali reserve* *This could mean approaching death*
4.	Bile salt (sodium glycocholate)	*In obstructive jaundice*
5.	Bile pigment (bilirubin glucuronide)	*Found in any form of hepatitis involving destruction of liver cells and obstructive jaundice*
6	Blood	*Hematuria-RBC in urine-due to trauma by catheter* *Hemoglobinuria-in burns, incompatible blood transfusion*

3.4.1. Analysis of Abnormal Urine

Reagents.

1. 1% Acetic acid
2. Strong NH_4OH (Liquor ammonia)
3. Ammonium sulphate (solid)
4. 5% Sodium nitroprusside (fresh)
5. Hydrogen peroxide
6. Sulphur powder
7. 10% Ferric chloride
8. Benedict's qualitative reagent. Dissolve 173 gm sodium citrate and 100 gm sodium carbonate in about 800 ml water with aid of heat Dissolve 17.3 gm copper sulphate in 100 ml water Add separately copper sulphate solution slowly with stirring to the carbonate-citrate solution and make upto 1 litre.
9. Benzidine solution 12% in glacial acetic acid.
10. Fouchet's reagent· Mix 10 ml of 10% $FeCl_3$ with 100 ml of 25% trichloroacetic acid

Table VII-24. Tests for analysis of abnormal urine

Constituents	*Test detail*	*Observation*
1 Albumin	*Heat and acetic acid test*	
	Take 10 ml urine in a test tube	*A cloudiness due to coagulated albumin*
	Hold it over a flame in a slanting position.	
	Boil the upper 5 ml portion (lower half serves as 'control')	
	Add a few drops of acetic acid.	
2. Glucose	*Benedict's test*	
	To 5 ml Benedict's reagent add exactly 8 drops of urine	*A light green colour indicates 0 5% glucose, a yellow colour, 1%, a brick-red colour, 2%*
	Boil for 1 to 2 minutes	
3 Ketone bodies	*Rothera's test for acetone*	
	To 5 ml urine add solid ammonium sulphate a little at a time with mixing to salurate the solution	*A purple ring is formed*
	Add 2 or 3 drops of sodium nitroprousside.	
	Mix well and add 1 ml strong ammonium hydroxide dropwise along the side.	
	Gerhardt's test for acetoacetic acid	
	To 3 ml urine add ferric chloride, drop by drop	*A port-wine colour is obtained*
	Filter the ferric phosphate formed	
	To the filtrate add ferric chloride	
	Note Salicylic acid formed in the degradation of aspirin interferes in this test To differentiate between the two perform the above test with a well-boiled sample of urine. The colour due to acetoacetic acid disappears	
4 Bile salt	*Hay's test*	
	To 2 ml urine, sprinkle fine sulphur powder.	*Bile salt decreases surface tension*
	Observe without mixing	*Sulphur sinks to the bottom*
5 Bilirubin	*Fouchet's test*	
	To 10 ml urine add 5 ml $BaCl_2$ and a pinch of $MgSo_4$	*Wet barrium sulphate adsorbs yellow bilirubin.*
	Mix well $BaSo_4$ is precipitated	*Fouchet's reagent oxidises*
	After 5 minutes filter the solution.	*bilirubin to green biliverdin*
	Unfold the filter paper over one or two filter papers, add few drops of Fouchet's reagent	
6 Blood	*Benzidine test*	
	To 3 drops benzidine add 2 drops H_2O_2	*A blue or green colour is*
	Add 1 drop of this to 2 ml urine.	*formed (unstable)*

3.4.2 Estimation of Albumin in Urine

Apparatus

Esbach's albuminometer

Apparatus. Esbach's albuminometer is a test tube like apparatus with the mark 'U' near the middle and the mark 'R' near the top. The portion below 'U' is graduated from 0-12 that gives the quantity of proteins in gm/litre.

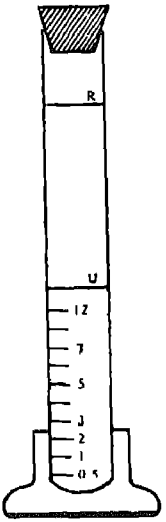


Fig. VII.17. ESBACH'S ALBUMINOMETER

Albumin and other proteins which are excreted in urine in disease conditions, can be measured by precipitating with the reagent in the apparatus.

Reagent:

Esbach's reagent: One gm picric acid and 2 gm citric acid in 100 ml water.

Procedure:

Fill the tube with urine upto 'U' (if urine has a high specific gravity it should be diluted so that it is around 1.008).

Add Esbach's reagent upto mark 'R'.

Stopper the tube. Mix by inversion several times.

Allow to stand (usually for 24 hours)

Read the calibration corresponding to the meniscus of the precipitate Express the value as gm/litre.

Significance

Normal urine contains only traces of proteins. Benign and transient proteinuria found in young people may be due to severe exercise Orthostatic proteinuria is apparantly due to the erect posture for prolonged periods

Albuminuria is characteristic of kidney diseases like acute and chronic nephritis, nephrotic syndrome, renal infections, poisoning by heavy metals and polycystic kidney In nephrotic syndrome 10-15 gm protein may be lost daily

3.4.3 Determination of Urine Sugar (Benedict's Method)

Reagents

1. Benedict's quantitative reagent.

Dissolve 200 gm sodium citrate, 75 gm anhydrous sodium carbonate and 125 gm potassium thiocyanate in about 600 gm water by gentle heating Filter the solution. Dissolve 18 gm $CuSO_4.5H_2O$ in 100 ml water separately and add this solution to filtrate slowly with stirring. Add 5 ml of 5% potassium ferrocyanide solution and make up the volume to one litre. Filter the solution if it is not clear.

2. Anhydrous sodium carbonate.

3 Porcelain bits.

When the qualitative test performed with urine and Benedict's qualitative reagent gives a brick red colour, exact quantity of glucose excreted in urine is estimated by this method. In this titrimetric procedure the reducing property of glucose is made use of. Benedict's quantitative reagent differs from the qualitative reagent in that it contains potassium thiocyanate and potassium ferrocyanide. Potassium ferrocyanide helps to keep in solution the cuprous oxide formed.

Glucose reduces copper sulphate in the reagent to cuprous oxide It in turn reacts with potassium thiocyanate to form a white precipitate of cuprous thiocyanate. Thus when the last traces of copper sulphate are converted to cuprous thiocyanate, the colour of

the solution will change from blue to white which is the end point of titration

Procedure.

Pipette 10 ml Benedict's quanitative reagent into a 100 ml conical flask.

Add 100 ml distilled water.

Add about 2 spatula of anhydrous sodium carbonate to provide the required alkalinity and a few porcelain bits

Bring the solution to boil and add urine from a burette Add urine rapidly in the beginning until a chalk white precipitate appears and the blue colour begins to fade

Thereafter, add urine drop by drop till the last traces of blue colour disappear The contents of the flask should be kept moderately boiling throughout the operation.

Calculation·

Benedict's quanitative reagent is so made that 20 mg glucose will reduce all the copper present in 10 ml reagent If 5 ml urine are required in the titration to reduce completely all the copper in 10 ml reagent, then this volume of urine should contain 20 mg glucose. Therefore, 100 ml urine will contain,

$$\frac{20 \times 100}{5} = 400 \text{ mg}$$

Significance·

Glycosuria occurs mainly during diabetes mellitus and in renal diabetes.

Note: This titrimetric procedure can also be used in the estimation of lactose in milk.

3.5 Additional Tests in Urine

In addition to the tests enumerated earlier, a few more substances are found in some physiological and pathological states. These are lactose, pentoses, fructose, homogentisic acid, phenylpyruvic acid (Table VII-25)

TABLE VII-25 *Constituents of urine found in specific conditions*

	Constituent	*Condition*
1	Lactose	This occurs in majority of cases of normal lactation
2	Pentoses	Alimentary penstosuria—after ingestion of cherries and plums in excess
		Essential pentosuria—a familiar hereditory condition.
3	Fructose	Alimentary fructosuria—after ingestion of honey and grapes
		Essential fructosuria—an inborn error of metabolism
4	Homogentisic acid	Alkaptonuria—an inborn error of incomplete tyrosin metabolism—a benign condition
5	Phenylpyruvic acid	Phenylketonuria—an inborn error of phenylalanine metabolism of clinical importance

The details of performance of tests for the detection of the above constituents in ruine are given in the Table VII-26

TABLE VII-26 Additional tests in urine

1	Lactose	*Methylamine Test* To 5 ml urine add 1 ml of Methylamine hydrochloride (2 g/l in water) and 0 2 ml 10% NaOH. Heat for 30 minutes at 50-60°C. Cool at room temperature If necessary, run a 'control' taking 5 ml of water in place of 5 ml of urine	Red color is got, Compare the color with control
2	Pentose	*Bial's Test* To 5 ml of Bials reagent add 0 5 ml of urine Heat to boil. Avoid boiling for longer than 30 seconds (Bial's Reagent 300 mg orcinol in 100 ml con HCl to which add 5 drops of 10% ferric chloride sol)	Green color is got.
3	Fructose	*Selwanoff's Test* To 0 5 ml urine add 5 ml of selwanoff's reagent and boil for 30 seconds (Selwanoff's Reagent 50 mg orcinol in 33 ml con HCl is diluted to 100 ml with water)	Red color is produced
4	Homogentisic acid	(a) Observe the urine Urine on standing gradually darkens in color from the surface towards the bottom	
		(b) To 0 5 ml of urine add a few drops of dilute ammonia and 5 ml of 3% silver nitrate sol.	A black colour is produced
		(c) To a few ml of urine add a few drops of 10% ferric chloride sol	A temporary blue or green color is produced
5	Phenylpyruvic acid	To a few ml of fresh urine add a few drops of 10% ferric chloride sol	A blue or green color produced gradually fades away

PART IV

DIAGNOSTIC TESTS IN BLOOD

4.1 Collection and Preservation of Blood, Serum and Plasma

The majority of tests carried out in clinical laboratory is on Blood, Serum and Plasma

Blood is collected mixed with some chemicals which prevent clotting. These chemicals are called anticoagulants The anticoagulants used are 1) Potassium oxalate, 2) Double oxalate of Na and K, 3) Sodium citrate, 4) Heparin, 5) EDTA (Ethylene diamine tetra acetate).

Venous blood is always collected

Whole blood is used where the concentration of a constituent estimated is distributed equally in the cells and plasma and that the level in blood or plasma is same. Eg: Blood sugar, urea etc. Otherwise serum and plasma is perferred.

Separation of plasma

Blood is collected in an anti-coagulant bottle and centrifuged. A clear straw or yellow fluid separates This is plasma

Serum

Blood is collected in a plain bottle (without any anticoagulant). Allowed to stand to form clot which takes about 30 minutes The clot is separated and the fluid is transferred to a centrifuge tube. It is centrifuged The serum is transferred with the help of pasteur pipet tc a bottle or tube.

The Table VII-27 gives the amount of anticoagulants to be used.

Table VII-27 Amount of anticoagulant to be used:

No	*Anticeagulant*	*Amount to be used*
1	Heparin (Na or K salt)	*2 mg/10 ml of blood*
2	EDTA (Dipotassium salt)	*10-20 mg/10 ml of blood*
3.	Potassium oxalate	*20-30 mg/10 ml of blood*
4.	Sodium citrate	*30 mg/10 ml of blood*
5	Sodium fluoride*	*10 mg/ml of blood*

***Sodium fluoride mixed with potassium oxalate in the collection of blood of sugar estimation. Fluoride prevents conversion of glucose to lactic acid**

Note:

Some of the constituents estimated show high values in hemolysed serum. So care is taken to avoid hemolysis. Thus, it is important that the syringe and needle are perfectly clean and dry. Mixing of blood with anticoagulant is done smoothly but not violently. Blood is emptied from syringe after the needle is removed and minimum pressure is put on the piston Avoid using excess of anticoagulants

Preparation of anticoagulant bottles·

Prepare a 30% potassium exalate solution (30 g in 100 ml water) Adjust the pH to 7 4 using potassium hydroxide or oxalic acid. Add 0.1 ml (to 10 ml) of blood in a bottle. Rotate the bottle all over as to get a thin film of it on the walls of bottle. Keep in hot-air-oven at 60.80°C for half an hour to dry The bottle now is ready for use.

4.2 Carbohydrates

4.2.1 Determination of Blood Sugar (Glucose)

4 2 1 1 Method Folin-Wu

Principle

The sugar of blood is glucose. Glucose reduces alkaline copper reagent Phosphomolybdic acid added develops blue color The optical density of blue color is measured in a colorimeter using blue filter (490 mm).

Blood Specimen

Venous blood is collected in an oxalate-fluoride bottle (To the oxalate bottle prepared as said earlier is added about 10 mg of sodium chloride)

Reagents

1 Sodium tungstate, 10%—Dissolve 10 g of sodium tungstate in about 70 ml water. Then make upto 100 ml with water.

2 0.667 N sulfuric acid Refer expt on 'Titrations'

3 Alkaline copper reagent—Dissolve 40 g anhydrous sodium carbonate in 400 ml water Add 7 5 g tartaric acid and 4 5 g copper sulfate Dissolve Make upto one litre with water.

4 Phosphomolybdic acid—Prepare 200 ml 10% sodium hydroxide solution Add to this 35 g molybdic acid and 5 g sodium tungstate Add 200 ml water. Boil till no smell of ammonai is got. Cool. Transfer to 500 ml flask Add carefully 125 ml syrupy phosphoric acid, keeping the flask cooled under tap. Make upto 500 ml with water.

5 Stock standard glucose solution—100 mgs per 100 ml. Boil

about 120 ml water. Add 2-3 spatual benzoic acid Mix Cool and filter Transfer exactly 100 mgs glucose (AR grade) to a 100 ml flask. Add the above solution upto 100 ml mark Mix thoroughly

6. Standard glucose solution for use 10 mgs per 100 ml Dilute the above stock 10 ml solution to 100 ml with water This gives 1 ml = 0 1 mg solution (Prepare fresh every day)

Procedure

Pipet 7 ml distilled water into a clean and dry 100 ml conical flask

Pipet 1 ml blood into the flask. Rotate well

Add 1 ml 10% sodium tungstate.

Mix.

Add 1 ml 0.667 N sulfuric acid. Mix Wait for 10 minutes

Filter through dry filtrate in a dry test tube

Label three Folin sugar tubes as B, S and T.

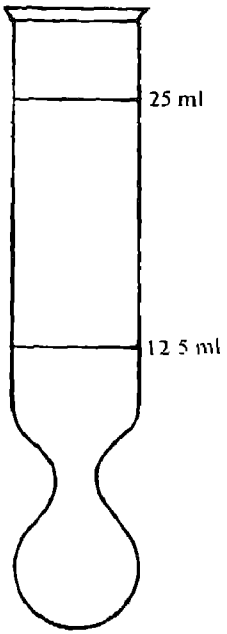


Fig VII-18 FOLIN-SUGAR TUBE

Keep ready boiling water bath Into B transfer 2 ml water, into S transfer 2 ml standard glucose for use and into T transfer 2 ml protein-free filtrate

Add 2 ml alkaline copper reagent to each of B, S & T

Keep all the three tubes in boiling water bath for exactly 8 minutes. Remove and cool.

Add 2 ml phosphomolybdic acid to each of B, S & T

Mix well by lapping against the plam.

Add water upto 25 ml mark in all the three tubes.

Mix by inversion

Read optical densities of B, S and T in a colorimeter using blue filter or at 490 nm The method is summarised in Table VII-27.

Table VII-27

Reagents	*B*	*S*	*T*
Distilled water	—	—	7
Blood	—	—	1
Sodium tungstate 10%	—	—	1
Sulfuric acid 0 667 N	—	—	1
Mix Wait 10 minutes Filter			
Filtrate	—	—	2
Distilled water	2	—	—
Standard glucose for use	—	2	—
Alkaline copper reagent	2	2	2
Keep in boiling water for 8 minutes, cool			
Phosphomolybdic acid	2	2	2
water upto	25 ml	25 ml	25 ml
O.D. (Optical Density) Blue filter or 490 nm			

Calculation

$$\text{mgs glucose per 100 ml blood} = \frac{\text{Reading of T} - \text{Reading of B}}{\text{Reading of S} - \text{Reading of B}} \times 100$$

Results

Glucose level in blood = mgs/100 ml

Note:

1 Water should boil vigorously before placing tubes.

2. Period of boiling is exactly 8 minutes.

Interpretation.

Fasting blood sugar level ranges from 80-120 mgs/100 ml Increases are soon in diabetes mellitus The condition is called hyperglycemia

Hyperglycemia is also seen in hyperthyroidism, emotional states like fear, anger and anxiety.

Decreases in blood glucose level is called hypoglycemia It is seen in tumours of pancreas. It is also seen in overdosage of insulin

4 2.1 2 Method O-Toluidine

Principle.

Glucose when heated with ortho-toluidine in acetic acid forms a blue-green colored compound which is measured photometrically.

Blood Specimen.

Same as given under Folin's method.

Reagents.

1 Ortho-Toluidine-Reagent.

To 5 0 g thiourea (AR grade) add 90.0 ml ortho-toluidine and dilute to 1 litre with glacial acetic acid. Store in brown bottle At refrigerator temperature the reagent is stable for a year.

2 Trichloro acetic acid, 10%:

Dissolve 10 g TCA (Trichloroacetic acid) in water and make up the volume to 100 ml.

3. Glucose standard

Prepare 100 mgs per 100 ml glucose solution in saturated benzoic acid as given under Folin's method.

Procedure

Blank. To 1 4 ml water add .0 6 ml 10% TCA. Mix well Transfer 1 ml to a test tube labeled B

Test To 1 2 ml water add 0 2 ml blood and mix Add 0 6 ml 10% TCA Mix well Wait for 5 minutes. Centrifuge for 10 minutes.

Transfer 1 ml clear supernatant to a test tube labeled T

Standard To 1 2 ml water add 0 2 ml glucose standard solution followed by 0 6 ml 10% TCA Mix well Transfer 1 ml to a test tube labeled S

To all the three tubes add 5 ml each of orthotoluidine reagent and mix

Keep them in boiling water bath for exactly 10 minutes Cool Read optical densities of B, S and T using red filter or at 630 nm

The method is summarised in Table VII-28

Table VII-28

Reagents	*B*	*S*	*T*
Distilled water	1 4 ml	1 2 ml	1 2 ml
Standard glucose solution	—	0 2 ml	—
Blood	—	—	0 2 ml
TCA, 10%	0.6 ml	0 6 ml	0.6 ml
Mix Wait for 5 minutes Centrifuge for 10 minutes			
Supernatant	1 0 ml	1.0 ml	1 0 ml
O-Toluidine	5 0 ml	5 0 ml	5.0 ml
Keep in boiling water for 10 minutes			
Optical Density (O D) Red filter or 6 30 nm			

Calculation

$$\text{mgs glucose per 100 ml} = \frac{\text{O D. of T} - \text{O D of B}}{\text{O D of S} - \text{O.D of B}} \times 100$$

Note

1 O-toluidine is highly corrosive. Handle carefully or preferably use gloves
2 Use automatic dispensers for O-toluidine reagent Do not pipet through mouth
3 The method is directly applied for cerebrospinal fluid and urine
4 Read the absorbance immediately

Interpretation

This method gives true glucose level whereas Folin's method includes other reducing substances in blood (like glutathione) The

normal range is between 60 to 100 mgs/100 ml blood It is increased in diabetes mellitus and decreased in insulin overdosage

Fasting blood sugar by this method ranges from 60-90 mgs per dl

Post-prandial (2 after food) blood sugar level ranges from 90-110 mgs/dl

Random blood sugar (anytime of day) level can be between 60-100 mgs/dl.

4.2.2 Glucose Tolerance Test (GTT)

The test is performed to assess the ability of the body to utilise an additional load of glucose given orally. The glucose given is utilised faster in normals than in diabetics. There is delay in removal of glucose in diabetics This is due to lack of insulin A comparison of the tolerance curve of patient with that of normal one gives an idea of the severity of diabetes.

Patient preparation·

Instruct the patient to make usual diet for at least three days preceeding the test

On the day previous to the performance of test instruct the patient not to take anything, except water, after 8 O'clock in the night. Ask the patient to come to the laboratory the next morning at 8 O'clock This means, the patient is on fasting for 8-12 hours before test.

Performance of test.

Collect venous blood at 8 O'clock. Label it as 'fasting sample' Collect urine sample at the same time Label as 'fasting urine'.

Dissolve 50 g of glucose (or 1 75 g/kg for children) in about 200 ml of water, flavour it if necessary. Ask the patient to drink glucose solution Note the time Collect blood and urine samples at intervals of 30, 60, 90 and 120 minutes. Label the blood samples and urine samples as 30,60 90 & 120 minutes samples respectively. During the period of performance of test (8-10.30 am) instruct the patient to be at rest and avoid smoking.

Samples·

Table VII-29 Samples of collection of blood and urine.

S No.	*Blood*	*Urine*
1	Fasting	Fasting
2	30 minutes	30 minutes
3	60 minutes	60 minutes
4	90 minutes	90 minutes
5	120 minutes	120 minutes

Estimate glucose levels in blood samples by one of the methods dealt earlier

Perform Benedict's qualitative tests on the urine samples. Tabulate the values

Draw a curve on a graph sheet plotting mgs glucose/100 ml on y-axis and time on x-axis

Factors influencing GTT·

1 Dose of glucose
50 g of glucose in 200 ml water seems adequate Larger dose will not improve diagnostic accuracy

2 Capillary or venous blood
There is not much difference in values of glucose in normal. But during high glucose level capillary blood shows higher levels than that of venous ones

3 Age.
With increasing age there is deterioration of glucose tolerance and mild impairment is less significant in older than in younger people

4 Diet
Restricted carbohydrate diet days before the test may show normal curve in mild diabetics

5. Time of day
Test conducted in the afternoon show higher values and the accepted normal may not be applicable.

Observations:

(a) Normal subjects (one example)

Table VII-30

Sample No	*mgs glucose/100 ml blood*	*Urine glucose*
Fasting	100	Negative
30 minutes	130-140	Negative
60 minutes	160	Negative
90 minutes	140	Negative
120 minutes	100	Negative

(b) Mild diabetics (one example)

Table VII-31

Sample	*mgs glucose/100 ml blood*	*Urine glucose*
Fasting	120	Negative
30 minutes	160	Negative or positive
60 minutes	200	Positive
90 minutes	180	Positive or Negative
120 minutes	140	Negative

(c) Severe diabetic (one example)

Table VII-32

Sample No	*mgs glucose/100 ml blood*	*Urine glucose*
Fasting	220	Positive
30 minutes	320	Positive
60 minutes	375	Positive
90 minutes	320	Positive
120 minutes	280	Positive

Criteria:

(a) Normal response—Refer 'normal subject' Table

1. The fasting blood sugar level is within the upper limit of 120 mg/100 ml.
2. The maximum level is reached within 30-60 minutes. This is always less than the renal threshold for glucose of 180 mgs/100 ml blood.

3. The insulin action is seen after 60 minutes as evidenced by the fall in blood glucose
4 At the end of 2 hours the blood glucose dips to the normal value of 100 mgs
5 None of the urine samples show positive reaction with Benedict's reagent

(b) Mild diabetics: This condition may be due to small deficiencies of insulin secretion

1 The fasting level is around the upper limit of 120 mgs/100 ml The glucose absorption is seen by the elevated levels of glucose between 30-60 minutes interval. The glucose levels may be around 200 mg during this period
2. The insulin response is weak as evident by a small dip in glucose level
3. The glucose level at the end of two hours is above normal.
4 At least one of the urine samples show positive reaction with Benedict's reagent

(c) Severe diabetic

1. The fasting level is above 200 mgs because of marked lack of insulin secretion
2. The glucose level crosses 300 mgs/100 ml in 30-60 minutes
3 The fall in glucose is very small due to the lack of insulin.
4. All the urine samples show positive reaction with Benedict's reagent

4.3 Non-Protein Nitrogenous Compounds

4.3.1 Determination of Blood Urea

4.3.1.1.Method I: Direct Nesslerisation.

Specimen.

Venous blood is collected. Use 2-3 mg/ml of potassium oxalate as anticoagulant

Principle:

The urease enzyme hydrolyses urea to ammonium carbonate which on treatment with Nesslere's reagent produces yellowish orange color. The absorbance of the color produced is measured photometrically using green filter or at 540 nm.

Reagents:

1 Sodium tungstate, 10%
Dissolve 100 g sodium tungstate in water and make upto one litre.

2 0 667 N sulfuric acid.
Dilute 10 ml of concentrated sulfuric acid in 500 ml water Determine the normality by titrating against 0 667 N sodium carbonate. Adjust the normality to 0.667 N by dilution.

3 Urease suspension:
Grind 1 g horse gram powder with 10 ml water and 500 mgs of potassium chloride. Use supernatant. Keep in refrigerator. Stable atleast a week

4 Nessler's reagent
Into a 500 ml vulmetric flask add 50 g mercuric iodide, 35 g potassium iodide and 200 ml distilled water Mix to dissolve.

In a beaker dissolve 50 g sodium hydroxide in 250 ml water. Add this solution to volumetric flask with stirring and

make upto 500 ml with water. Use only the clear supernatant. The reagent is stable stored in polythene bottles

5. Standard urea solution, 60 mgs per 100 ml
   Dissolve exactly 60 mgs urea, AR grade in water and make upto 100 ml Store in refrigerator Prepare fresh every weak

Procedure

Label two centrifuge tubes as T and S

Into T pipet 0 5 ml blood and 3.0 ml water

Into S pipet 0 5 ml standard and 3.0 ml water.

Incubate at 55°C for exactly 15 minutes

Add 0 5 ml each of urease suspension to T and S. Note the time

Incubate at 50°C for exactly 5 minutes

Stop enzyme reaction by adding 0 5 ml of 0 667 N sulfuric acid to T and S Mix. Stand for 3 minutes.

Add 0.5 ml of 10% sodium tungstate to T and S. Mix.

Centrifuge at 2000 rpm (Rotation per minute) for 5 minutes.

Label three test tubes as B, S and T. Into B pipet 7 ml water Into S pipet 2 ml supernatant from centrifuge tube S and add 5 ml water Into T pipet 2 ml supernatant from centrifuge tube T and add 5 ml water

Mix all the tubes.

Add 1 ml Nessler's reagent to B and take reading, using green filter or at 540 nm in a colorimeter.

Add 1 ml Nesslers reagent to T and take reading.

Add 1 ml Nessler's reagent to S and take reading.

The steps in the procedure are summarised in Table VII-33

Table VII-33

Reagents	*B*	*S*	*T*
Water	—	3 ml	3 ml
Blood	—	—	0 5 ml
Standard urea	—	0 5 ml	—
Urease suspension	—	0 5 ml	0 5 ml
Incubate at 55°C for 15 minutes			
Sulfuric acid, 0.667 N	—	0 5 ml	0 5 ml
Mix Stand for 3 minutes			
Sodium tungstate, 10%	—	0 5 ml	0 5 ml

Mix centrifuge for 5 minutes			
Supernatant	—	2 ml	2 ml
Water	7 ml	5 ml	5 ml
Nessler's reagent	1 ml	1 ml	1 ml
O D (Green Filter) or at 540 nm			

Calculation:

mgs urea per 100 ml

$$= \frac{\text{Reading of T} - \text{Reading of B}}{\text{Reading of S} - \text{Reading of B}} \times 0.12 \times \frac{100}{0.2}$$

$$= \frac{\text{Reading of T} - \text{Reading of B}}{\text{Reading of S} - \text{Reading of B}} \times 60$$

Note.

1. Ammonium oxalate should not be used as an anticoagulant.
2. Do not mix after adding nessler's reagent. It may form turbidity.
3. To avoid turbid formation add one drop of saturated sodium potassium tartarate solution.
4. If turbidity is got due to high content of urea, repeat the color development step (Nesslor's reagent addition) taking 1 ml supernatant. Apply dilution factor in calculation.

4.3.1 2.Method II. Diacetyl Monoxime

Principle:

Urea reacts with diacetyl monoxime under strongly acidic conditions in presence of ferric ions and thiosemi-carbazide to give pink colored complex.

Specimen:

Oxalated venous blood or serum.

Reagents:

1. Diacetyl monoxime.
   Dissolve 1.56 g diacetyl monoxime in 250 ml water.
2. Ferric chloride:

Dissolve 324 mgs of ferric chloride in 10 ml of 56% orthophosphoric acid. Store in brown bottle.

3. Thiosemicarbazide:
Dissolve 41 mgs of thiosemicarbazide in 250 ml of water.

4 Sulfuric acid, 20%.
Add 200 ml of concentrated sulfuric acid to 800 ml of water in a beaker slowly with stirring and cooling.

5. Acid reagent.
To 1 litre of 20% sulfuric acid (Reagent 4) add 1 ml of ferric chloride reagent (Reagent 2)

6 Trichloroacetic acid, 10%:
Dissolve 10 g of TCA in water and make upto 100 ml

7. Preservative diluent for standard.
Heat about 250 ml water and add 40 mgs of phenyl mercuric acetate. Mix to dissolve. Transfer to 1 litre graduated cylinder. Add 0 3 ml concentrated sulfuric acid and make up the volume to 1 litre and mix

8 Stock standard urea, 0.5 mg per ml
Dissolve 50 g of urea (GR grade) in 100 ml of preservative diluent (Reagent 7)

9 Standard urea for use. 0 1 mg per ml
Dilute 1 ml of stock standard (Reagent 8) solution to 50 ml with preservative diluent (Reagent 7).

Procedure:

Test Take 3.4 ml of water in a test tube and add 0.1 ml blood and mix

Add 1.5 ml of 10% TCA Mix and let stand for 5 minutes.

Centrifuge at 2000 rpm for 5 minutes Lable a test tube as T and pipet 1 ml supernatant from above

Standard· Take 1 ml of standard urea for use in a test tube labeled S

Blank: Take 1 ml water in a test tube labeled B

Add 1 ml each of diacetyl monoxime to B, S and T.

Add 1 ml of thiosemicarbazide, followed by 3 ml of acid reagent to B, S and T. Mix thoroughly

Place the three tubes in a boiling water bath for exactly 15 minutes. Cool

Read absorbances of B, S and T using a green filter or at 540 nm setting the reading to zero with water

The Procedure is summarised in Table VII-34.

Table VII-34

Reagents	*B*	*S*	*T*
Water	—	—	3 4 ml
Blood	—	—	0 1 ml
TCA, 10%	—	—	1.5 ml
Mix Wait for 10 minutes Centrifuge			
Supernatant	—	—	1 0 ml
Water	1 0 ml	—	—
Standard urea for use	—	1 ml	—
Diacetyl monoxime	1.0 ml	1.0 ml	1 0 ml
Thiosemicarbazide	1 0 ml	1.0 ml	1.0 ml
Acid reagent	3 0 ml	3 0 ml	3 0 ml
Place in boiling water both for 15 minutes and cool			
O D (Blue filter or 540 nm)			

Calculations:

$$\text{mgs of urea in 100 ml blood} = \frac{\text{O.D. of T} - \text{O D. of B}}{\text{O.D of S} - \text{O D of B}} \times 0\,01 \times \frac{100}{0\,2}$$

$$= \frac{\text{O D of T} - \text{O.D of B}}{\text{O D of S} - \text{O D. of B}} \times 50$$

Note:

1. Arrange boiling water bath near the window because it gives out foul smell.
2. The urea standards can be prepared in water. This is stable for about 5 days when refrigerated.
3. If chemicals are available this method is superior to Nessler's
4. Boiling in water bath should be for exactly 15 minutes.
5. Small differences in acid reagent will cause differences in O D.

6. If the O.D. of T is high, dilute the solution and read Apply dilution factor in calculation

Interpretation·

Normal blood urea ranges from 15-45 mgs per 100 ml It is dependent on proteins in the diet It is lower on low protein diets. Increases are seen in—

1 Hemo concentration due to sever vomiting and diarrhoea and in burns
2. In renal diseases (renal failure)
3. High protein diet
4 Fever, cancer.

4.3.2 Determination of Creatinine in Serum or Plasma

Method: Bonsnes and Tausky

Specimen· Serum or plasma

Principle·

Creatinine in presence of alkali forms alkaline creatinine picrate. The red color so formed is measured colorimetrically and creatinine is determined

Reagents

1 Sulfuric acid 0.667N.
Dilute 10 ml of concentrated sulfuric acid to 500 ml water. Determine the normality by titrating against 0 667 N sodium carbonate. Adjust the normality to 0.667 N by dilution.

2. Sodium tungstate, 5%.
Dissolve 5 g of sodium tungstate in water and make upto 100 ml

3. Picric acid, 0.04 M
Dry some picric acid crystals by putting over a pad of filter paper Dissolve 9.16 g of dry picric acid in water and make up to one litre

4 Sodium hydroxide, 0.75 N
Weigh about 7 g of sodium hydroxide and prepare 200 ml solution in water Determine the normality by titrating against

0 667 N sulfuric acid (Reagent 1). Adjust the normality of sodium hydroxide to 0.75 N by proper dilution.

5. Stock standard creatinine solution, 1 mg per ml.
   Dissolve 100 mgs of creatinine in 0.1 N HCl (1 ml concentrated HCl diluted to 100 ml) and make upto 100 ml with acid. Store in refrigerator Stable for one month.

6. Standard creatinine for use: 0.04 mg per ml
   Dilute 4 ml of the stock standard to 100 ml with water.

Procedure:

Pipet 2 ml of water into a centrifuge tube labeled T.
Add 2 ml of serum.
Add 2 ml of 0.667 N sulfuric acid Mix.
Add 2 ml of 5% sodium tungstate.
Mix and stand for 10 minutes.
Centrifuge or filter.
Label another centrifuge tube as standard(s).
Add 2 ml of water and 2 ml of standard for use solution
Label a third centrifuge tube as Blank (B).
Add 4 ml of water to (B)
Now add 2 ml of 0.667 N sulfuric acid and 2 ml of 5% sodium tungstate to each of S and B Mix
Mark three test tubes as Test T, Standard S, and Blank B.
Pipet 3 ml of supernatant from centrifuge tube T to test tube T.
Pipet 3 ml of solution from centrifuge tube B to test tube B, and 3 ml of sol from centrifuge tube S to test tube S.
Add 1 ml of picric acid to each of T, S and B.
Add 1 ml of 0.75 N sodium hydroxide to each of T, S and B.
Mix.
Stand for 15 minutes.
Read optical densities of B, S, and T after setting the colorimeter to zero with water, using green filter or at 520 nm.
The procedure is summarised in Table VII-35.

Table VII-35

Reagents	*B*	*S*	*T*
Water	4 ml	2 ml	2 ml
Serum	—	—	2 ml
Standard for use solution	—	2 ml	
Sulfuric acid, 0 667 N	2 ml	2 ml	2 ml
Sodium tungstate, 5%	2 ml	2 ml	2 ml
Mix Stand for 10 minutes. Centrifuge			
Supernatant	3 ml	3 ml	3 ml
Picric acid, 0 04 M	1 ml	1 ml	1 ml
Sodium hydroxide, 0.75 N	1 ml	1 ml	1 ml
Mix. Wait for 15 minutes			
Optical density (Green or 520 nm)			

Calculation

$$\text{mgs creatinine per 100 ml serum} = \frac{\text{O.D. of T} - \text{O.D of B}}{\text{O D. of S} - \text{O.D of B}} \times 0\,03 \times \frac{100}{0.75}$$

$$= \frac{\text{O.D of T} - \text{O.D. of B}}{\text{O.D. of S} - \text{O D of B}} \times 4$$

Interpretation.

Normal value ranges from 0 7-1 2 mg/100 ml serum. It is a little lower in women (0 5-1.0 mg/100 ml). Serum creatinine is raised in renal diseases, lowered in muscular dystrophy.

4.3.3 Determination of Uric Acid in Serum or Plasma

Method. Henry et al

Specimen Non-Hemolysed serum or plasma.

Principle:

In presence of sodium carbonate uric acid reduces phosphotungstic acid to a blue colored compound. The blue color developed is measured colorimetrically and the uric level is determined.

Reagents

1. Sulfuric acid 0.667 N:

Dilute 10 ml concentrated sulfuric acid to 500 ml. Determine the normality using 0 667 N sodium carbonate standard solution. Adjust the normality of acid to 0 667 N by dilution.

2 Sodium tungstate 10% (w/v).
Dissolve 10 g of sodium tungstate in water and make upto 100 ml

3. Sodium carbonate 14%·
Dissolve 14 g of sodium carbonate in about 80 ml of water and make up to 100 ml.

4. Phosphotungstic acid reagent:
Dissolve 40 g of sodium tungstate in 300 ml water in a litre round bottom flask. Add 32 ml of syrupy phosphoric acid (85%) and several glass beads or porcelein bits. Attach a reflux condenser and boil gently for 2 hours Cool to room temperature. Add32g of lithium sulfate and dissolve. Dilute to one litre. Store in amber color bottle. This reagent is stable indefinitely.

5 Stock standard uric acid, 1 mg per ml
Dissolve 60 mgs of lithium carbonate in 20 ml of water Heat the solution to about 60°C and add exactly 100 mgs of uric acid Stir until dissolved. Cool, transfer to a 100 ml volumetric flask with rinsing. Add 2 ml of formalin (40%) and 1 ml of 50% acetic acid solution Make upto 100 ml mark with water Mix Keep in a well stoppered bottle in dark or in refrigerator. The solution keeps at least for one month

6. Standard uric acid for use:
0.01 mg/ml.
Dilute 1 ml of the stock solution to 100 ml with water. Prepare the solution everyday.

Procedure:

Into a test tube pipet 8 ml of water
Add 1 ml of serum or plasma
Add 0.5 ml 0 667 N sulfuric acid.
Add 0.5 ml of 10% sodium tungstate.
Mix well.
Centrifuge for 10 minutes
Now, label 3 test tubes as Test (T), Standard (S) and Blank (B)
Into T, transfer 3 ml of supernatant.
Into S, transfer 3 ml of standard uric acid for use solution.
Into B, add 3 ml of water.

Add 1 ml of phosphotungstic acid reagent to each of T, S and B tube.

Add 1 ml of 14% sodium carbonate to each of T, S and B tube Mix

Let stand for 15 minutes

Read optical density using red filter or at 700 nm.

The method is summarised in Table VII-36

Table VII-36

Reagents	*B*	*S*	*T*
Water	—	—	8 0 ml
Serum or plasma	—	—	1 0 ml
Sulfuric acid, 0 667 N	—	—	0 5 ml
Sodium tungstate, 10%	—	—	0.5 ml
Mix Centrifuge for 10 minutes			
Supernatant	—	—	3.0 ml
Water	3.0 ml	—	—
Standard uric acid for use	—	3 0 ml	—
Phosphotungstic acid reagent	1.0 ml	1 0 ml	1 0 ml
Sodium carbonate, 14%	1 0 ml	1.0 ml	1 0 ml
Wait for 15 minutes			
O.D Red filter or 700 nm			

Calculation:

$$\text{mgs of uric acid per 100 ml serum} = \frac{\text{O D of T} - \text{O D of B}}{\text{O D. of S} - \text{O D. of B}} \times 0\,03 \times \frac{100}{0.3}$$

$$= \frac{\text{O D of T} - \text{O D of B}}{\text{O D of S} - \text{O D of B}} \times 10$$

Note:

The serum or plasma should be non-hemolysed.

Interpretation.

The normal level ranges from 3 to 7 mg per 100 ml In women it is 1 mg per 100 ml less than that in men

Uric acid increases in pneumonia, sepsis, leukemia, polycythaemia, anemias as well as in acute gout attacks It increases in renal failure

It is decreased in acromegaly.

4.4 Proteins

4 4.1 Determination of Serum Proteins and Albumin—Globilin Ratio

4 4.1 1. Method I Biuret Method

Specimen:

Serum is separated from the venous blood.

Principle.

Serum is treated with Biuret reagent to produce a pink color. The absorbance of the pink colored solution is read and total protein is estimated.

For the estimation of albumin, globulins are precipitated by sodium sulfite The filtrate contains albumin which is treated with Biuret reagent to develop a pink color Thus albumin is estimated.

From the difference of total proteins and albumin is found the globulin level

Globulin = Total proteins − Albumin

Reagents:

1. Sodium chloride, 0 9%
   Dissolve 900 mgs of sodium chloride in a little of water and make upto 100 ml.
2. 0 2 N sodium hydroxide
   Dissolve 8 g of hydroxide in about 400 ml of water in a litre flask. Make upto one litre
3. Biuret reagent.
   Dissolve 45 g of sodium potassium tartarate in 400 ml of 0.2 N sodium hydroxide (Reagent 2). Add 15 g of copper sulfate stirring continuously. Add 5 g of potassium iodide. Dissolve and make upto one litre with 0 2 N sodium hydroxide. This is the stock Biuret reagent Store in a polythene bottle It is stable for months

4. 0 2 N sodium hydroxide containing 5 g of potassium iodide per litre.
   Add 5 g of potassium iodide per litre to Reagent 2 and dissolve
5 Biuret reagent for use:
   Dilute 50 ml of stock Biuret reagent (Reagent 3) to 250 ml with 0 2 N sodium hydroxide containing 5 g potassium iodide per litre (Reagent 4)
6 Standard protein solution 6 mg/ml.
   Dissolve 714 3 mgs of Bovine albumin and 100 mgs of sodium azide used as preservative in 100 ml water. Store at 4°C in refrigerator
7. Sodium sulfite 28%.
   Dissolve 28 g of anhydrous sodium sulfite in about 70 ml of water Make up to 100 ml
8. Ether, AR grade

Procedure:

Into a centrifuge tube pipet 5.8 ml of 28 0% sodium sulfite. Transfer 0.2 ml serum from an ostwald pipet.

Add about 2 ml of ether.

Close the mouth of the centrifuge tube with thumb and mix gently for one minute.

Centrifuge at 2000 rpm for 5 minutes Globulin forms a coin like precipitate below the ether layer

Set up four test tubes as B (Blank), S (Standard) Tp (Total proteins) and A (Albumin)

Into S add 3 ml of standard protein solution.

Into A pipet 3 ml of clear fluid below the globulin layer, carefully. Into Tp pipet 2,9 ml of 0 9% sodium chloride and 0,1 ml of serum using ostwald pipet

To each of B, S and Tp and A add 3 ml of Biuret reagent for use. Mix. Let stand for 10 minutes.

Read optical densities of B, S, Tp and A in a colorimeter using green filter or at 540 nm, setting to zero with water.

The procedure is summarised in Table VII-37.

Table VII-37

Reagents	*B*	*S*	*A*	*Tp*
Sodium sulfite, 2 8%	—	—	5 8 ml	—
Serum	—	—	0 2 ml	—
Ether	—	—	2.0 ml	—
Mix gently Centrifuge for 5 minutes				
Clear supernatant	—	—	3 0 ml	2 9 ml
Sodium chloride, 0 9%	—	—	—	—
Serum	—	—	—	0 1 ml
Standard protein solution	—	3.0 ml	—	—
Water for 0.9% (NaCl)	3 0 ml	—	—	—
Biuret reagent for use	3 0 ml	3 0 ml	3 0 ml	3 0 ml
Mix. Stand for 10 minutes.				
O.D. Green Filter or at 540 nm				

Calculation.

$$\text{mg of total proteins per 100 ml} = \frac{\text{O.D. of Tp} - \text{O.D of B}}{\text{O D of S} - \text{O.D. of B}} \times \frac{100}{0.1} \times 6$$

$$\text{gm of total proteins per 100 ml} = \frac{\text{O.D. of Tp} - \text{O.D. of B}}{\text{O.D. of S} - \text{O D of B}} \times 6$$

$$\text{gm of Albumin/100 ml} = \frac{\text{O.D of A} - \text{O.D. of B}}{\text{O D of S} - \text{O.D. of B}} \times 6$$

Globulins = Total proteins — Albumin.

A G ratio is obtained by dividing albumin level by globulin level

Eg· Say,

Albumin = 4.4 g/100 ml

Globulin = 2 2 g/100 ml

Then, A · G Ratio = 4.4 . 2.2

= 2:1

Note:

1. Ether is used just to separate the globulin layer.
2. Mixing is very important to precipitate globulins. Vigorous mixing may precipitate albumin also Too gentle mixing may result in incomplete precipitation of globulins.
3. Globulins may be removed by filtering through Whatman No. 44 filter paper, in which case ether need not be added.
4 Keep flames away as ether is inflamable
5 Albumin concentration per gram of Bovine albumin is better determined by Kjeldahl method

Interpretation.

Total proteins averages to 7 g per 100 ml serum. The range is between 6.3 and 7.9 g per 100 ml.

Albumin ranges from 3 7 g to 5.3 g per 100 ml, globulin from 1 8 g to 3.6 g with albumin-globulin ratio of 2.5 1 to 1 2 1.

Increase in total proteins is seen in hemo-concentration due to dehydration.

A decrease in albumin is due to a low albumin level. Decreased albumin level or hypo-proteinemia is caused by protein loss through (i) heavy loss of a albumin or proteins in urine. The condition is called albuminuria or proteinuria. It causes edema which is seen in nephrotic syndrome;

(ii) Lead and Mercury poisoning,

(iii) Glomernlo nephritis, etc.

Hypoproteinemia is also seen in enteritis, colitis and in severe burns.

Hypoalbuminemia is seen especially in chronic liver disease cirrhosis and anaemia.

Protein deficiency in diet causes kwashiorkor.

4 4.1.2. Method II. Albumin by Dye-binding.

Principle:

Total protein in serum is estimated by the method of Biuret.

Serum Albumin: Albumin binds with bromocresol green (dye) in a suitable buffer to give a green color, albumin-bromocresol green compled. The green color produced is measured colorimetrically.

From the difference is found globulin level

Reagents

For total proteins refer Method I (Biuret Method)

For Albumin

1. Succinate buffer, 0.1 M, pH 4 0:
   Dissolve 11 9 g of succinic acid in about 800 ml of water Adjust the pH to 7.4 by adding IN NaOH (4g/100 ml), and dilute to one litre with water Store at 4°C, If 100 mg of sodium azide per litre of buffer is added, it keeps for at least one month
2. Bromocresol Green (Dye solution).
   stock solution, 0 6 nM—Dissolve 419 mg of BCG in 10 ml of O IN NaOH solution in one litre flask Dilute to one litre with water Store at 4°C or in refrigerator. The dye solution is stable for a month.
3. Working dye solution.
   Dilute 250 ml of stock BCG solution to one litre with 0 1 M succinate buffer Add 8 0 ml of 15% Brij-35 (15 g Brij in 100 ml water). Store at room temperature Prepare fresh every week
4. Standard protein solution, 6 mg per ml
   Dissolve 714.3 mg of bovine albumin (whose protein content is determined by Kjeldahl method), add 100 mg of sodium azide and make up to 100 ml with water. Store at 4°C. The reagent is stable
5. 0.9% NaCl solution (9 g of NaCl in 1 litre)

Procedure:

Into a test tube pipet 5 8 ml of 0 9% NaCl solution and 0 2 ml serum. Mix

Transfer 0.5 ml from this to a test tube labeled Test (T).

In another test tube pipet 2 5 ml of 0 9% NaCl and 0.5 ml of standard protein solution. Mix.

Transfer 0.5 ml from this to a test tube labeled Standard (S).

Transfer 2 5 ml of 0.9% NaCl and 0.5 ml of water to a test tube labeled Blank (B).

Add 5 ml bromocresol green solution to each of B, S and T. Mix

Stand at room temperature for 10 minutes.

Read the absorbance at 630 nm setting the colorimeter to read zero with water.

Calculation:

gms of albumin per 100 ml serum

$$= \frac{\text{O.D. of T} - \text{O D. of B}}{\text{O.D. of S} - \text{O D of B}} \times 3$$

Total protein − Albumin = Globulin

Note·

The method is simple, precipitation of globulin is dispensed with

4.4.2 Serum Protein Electrophoresis

This has been already delt under "Instrumentation".

4.4.3 Zinc Sulfate Turbidity Test

Method. Kunkel.

Sample: Serum.

Principle.

Globulin of the serum proteins produces a turbidity when mixed with buffered zinc sulfate solution.

Reagents:

1. Buffered zinc sulfate solution:
   Dissolve 24 mgs of zinc sulfate ($ZnSO_4$ $7H_2O$), 210 mgs of sodium barbitone, and 280 mgs of barbitone in CO_2-free water (double distilled water) and make up to one litre. Check the pH. It should be between 7.45 and 7.55, if not adjust by adding either barbitone or sodium barbitone solution
2. Barium chloride solution.
   Dissolve 1.15 g of barium chloride ($BaCl_2.2H_2O$) in water and make upto 100 ml Dilute 3 ml of this with 0.2 N sulfuric acid to 100 ml. The turbidity of this ($BaSO_4$ solution) is equivalent to 20 Kunkel units.

Procedure:

Pipet 0.1 ml of serum to 6 ml of buffered zinc sulfate solution Mix Stand at room temperature for 30 minutes. Shake and read the turbidity using red filter after setting 20 zero with water.

Read the turbidity of $BaCl_2$ in 0.2 N sulfuric acid the same way

Calculation:

$$\text{Zinc sulfate turbidity} = \frac{T_1}{S_1} \times 20 \text{ units}$$

Note:

Heparnised plasma is unsuitable

Interpretation:

Normal value ranges from 2-8 units. Increases in cirrhosis of liver

4.5 Electrolytes

4.5.1 Determination of Sodium and Potassium in Serum

Method: By means of Emaission flame photometry.

Sample. Strictly non-hemolysed serum

Principle:

Sample is diluted and fed into non-luminous flame in the form of fine spray. Sodium and potassium elements give out characteristic color in the flame Sodium gives yellow and potassium gives violet color on ignition in the flame Yellow or violet flame thus emitted is proportional to the sodium or potassium content. Separate color filters are used for sodium and potassium

Reagents:

1. Double distilled water The method is very sensitive. Water must be free of metal ions. It is better to use all-glass distilled double distilled water
2. Stock standard sodium 100 mEq/L
   Keep a little of AR grade sodium chloride at 110°C in an air oven for 2-3 hours. Allow to cool in a desiccator. Now, weigh 11 69 g of dried sodium chloride and dissolve in water and make upto one litre Mix thoroughly. Store in polythene bottle. Keeps at least for 2 weeks.
3. Stock potassium standard solution 10 mEq/L:
   Dry some potassium chloride crystals as given under standard sodium (Reagent 2) Weigh 0.746 g of dried potassium chloride and dissolve in a little of water and make upto one litre. Mix thoroughly. Store in polythene bottle. Keeps at least for 2 weeks

Working standards. A series of combined sodium-potassium standards are prepared. Mix sodium stock standard and stock potas-

sium standards in the volumes given in the Table below and make upto one litre in each instance

Proper labelling is important. Store in separate bottles

Table VII-38 Working sodium—potassium standards

Working std.	*Stock Na^+ (Sod) sol (ml)*	*Stock K^+ sol. (ml)*	*Na^+ content mEq/L*	*K^+ content mEq/L*
A	5.5	2.0	1 1	0.02
B	6.0	3 0	1 2	0.03
C	6 5	4 0	1 3	0 04
D	7 0	5 0	1.4	0 05
E	7 5	6.0	1 5	0 06
F	8.0	7 0	1 6	0.07
G	8 5	8.0	1 7	0 08

Procedure

Dilute the serum 1 in 100 with double distilled water. That is, dilute 0.2 ml of serum with 19 8 ml of water in a 100 ml conical flask. Mix thoroughly.

Take about 5 ml of working standard A (Na^+ — 1.1, K^+ — 0.2 mEq/L) in a 10 ml beaker labeled A. Likewise place 5 ml each of working standards from B, C, D, E, F & G in to 10 ml beakers labelled corespondingly as B, C, D, E, F and G.

Potassium.

Insert the K^+ filter in place in the flame-photometer.

Switch on the galvanometer light Turn on the knob for the air supply and regulate the air pressure to 12 lb per sq inch.

Turn the gas supply on and light the wick and obtain a non-luminous flame.

Take about 10 ml distilled water in a small beaker. Dip the end of the tubing connected to the spray intake. Adjust the gas supply in order to obtain non-luminous flame showing no yellow zone

Allow a few minutes for the instrument to warm up. Place working standards in the beakers A, B, C, D, E, F and G in order one after another in a row

Spray into the flame by dipping the tubing for the spray intake in water

Operate the zero control to return the galvanometer to zero.

Remove the spray intake tubing from water and dip in high K^+ standard i.e. beaker G. Adjust the sensitivity knob of the instrument to read 80 on the scale. Check zero with water and the standard again.

Then spray K^+ standard from B into the flame and note the galvanometer reading. Check with water for zero and the standard again.

Likewise note galvanometer readings of B, C, D, E, F and checking each time with water to zero and the standards

Now we have seven galvanometer readings corresponding to different standards.

Now, spray the diluted serum sample into the flame and note the reading.

Tabulate as follows: Serum diluted 1 in 100.

Table VII-39

Beakers	*K^+* content mEq/L	*Galvanometer Reading*
A	0 02	—
B	0 03	—
C	0 04	—
D	0 05	—
E	0.06	—
F	0 07	—
G	0 08	—
TEST		

Calculation:

It is well understood with an example. Reading of Test, say, lies in between the reading of Standards, C & D i.e. the concentration between 0 04 mEq/L and 0.05 mEq/L K^+, say

Reading of Test .. = 62

Reading of 0.05 mEq/L Standard (C) .. = 54

Reading of 0.05 mEq/L K^+ Standard (D) .. = 67

Difference in readings of C & D .. = 67 − 54 = 13 units

Difference in readings of Standard C & Test = 62 − 54 = 8

$$\text{Conc. of Test} = 0.04 \times \frac{8}{13} \times 0.01 \quad = 0\ 0406\ \text{mEq/L}$$

where 0.01 is the difference in K^+ content between C & D i.e. (0.05-0.04)

Concentration in serum = 0 460 × 100 = 4.60 mEq/L

where 100 is the dilution factor.

Sodium:

Remove K^+ filter and place Na^+ filter into the instrument. Repeat the above procedure with the same series of Standard & Test solutions Calculate in the same way as K^+.

Note:

1 Serum or plasma should be strictly free of hemolysis otherwise results deviate widely.
2. Blood is collected and serum is separated immediately after clot formation.
   If the analysis can not be done immediately keep the serum separated. But never refrigerate whole blood prior to separation of plasma.
3. Check the instrument for zero with water in between each standards

Interpretation.

Normal value	Sodium	138 – 146 mEq/L
	Potassium	3 8 – 5 2 mEq/L.

Sodium increases in dehydration, and in some cases of Cushing's syndrome. Increase in sodium is called hypernatraemia Hyponatraemia or lowered sodium level is found in diarrhoea, malabsorption

Potassium is increased in acute renal failure

Lowered potassium is found in diarrhoea, malabsorption syndrome, pyloric obstruction and in starvation.

4.5.2 Determination of Serum Chloride

Method: Schales and Schales.

Sample: Serum or plasma

Principle:

Serum is titrated with mercuric nitrate solution using diphenyl carbazone as indicator, which give a violet-blue color at the end point.

Reagent:

1. Mercuric Nitrate·
   Take 20 ml of double distilled water in a beaker. Add 3 ml concentrated nitric acid Add 3 2 g of mercuric nitrate to the solution in beaker. Dissolve Transfer the solution in the beaker to one litre flask and add water upto 1 litre mark Mix The reagent keeps indefinitely.
2. Nitric acid, approximately 1 N
   Dilute 6 ml of concentrated HNO_3 to 100 ml with water.
3 Diphenyl carbazone, 0.1%·
   Dissolve 100 mgs of diphenyl carbozone in 100 ml of 95% ethyl alcohol. Stored in a brown bottle in refrigerator, keeps indefinitely.
4 Ethyl ether.
5. Standard chloride solution 100 mEq/L.
   Dry some sodium chloride (AR grade) crystals at 110°C for 2-3 hours in a hot-air oven Allow it to cool in a desiccator.
   Weigh 5.845 gms of dry Nacl and dissolve in double distilled water and dilute to one litre

Procedure.

Test:

Into a 25 ml conical flask (or test tube) pipet 2 ml water.

Add 0.2 ml serum. Add 1 drop of 1 N nitric acid. Add 3 drops of diphenyl carbazone. Add 1 ml of ethyl ether.

Take mercuric nitrate solution in a 2 ml pipet calibrated 0.01 ml, upto '0' mark

Add mercuric nitrate to the conical flask (or test tube) drop by drop with shaking.

A pink color obtained in the beginning disappears. Continue addition till a violet-blue color, which remain permanent, is got. Note the titre value.

Standard:

Pipet 2 ml of water and 0.2 ml of standard into a 25 ml conical flask. Add one drop of 1 N nitric acid.

Add 3 drops of diphenyl carbazone.

Add 1 ml of ethyl ether

Titrate with mercuric nitrate to the permanent violet blue color.

Note the titre value.

Calculation:

$$\text{mEq/L of chloride} = \frac{\text{Titre Value of Test}}{\text{Titre Value of Standard}} \times 100$$

Note

1. There is no serious error if ether is not added.
2. Use double distilled water.
3. Sodium chloride should be dried before weighing.

Interpretation:

The normal level is between 94 and 111 mEq/L. Hyperchloraemia or an increase in serum chloride level is seen in dehydration and acute renal failure.

Hypochloraemia or lowered chloride level in serum is seen in diarrhoea, congestive heart failure, pyloric obstruction, uraemia, Addision's disease, pulmonary emphysema and diabetic acidosis.

4.5.3 Determination of Serum Calcium

4.5.3.1 Method: Trinder

Sample. Serum.

Principle:

Calcium is precipitated with naphthylhydroxamic acid. The precipitate is dissolved in EDTA and the color is developed with ferric nitrate. The absorbance of color compound is measured in a colorimeter and calcium level is determined

Reagents:

1. Calcium reagent

In a 250 ml beaker add 100 ml of water and 5 ml of ethanolam-

ine Add 2 g of tartaric acid. Mix. Add 250 mg of naphthylhydroxamic acid and dissolve by warming
In a 1 litre flask add 9 g of sodium chloride and 500 ml of water

Pour the contents of the beaker into the 1 L flask containing sodium chloride solution Add water upto 1 L mark. Mix. Filter through Whatman No 40 filter paper. The reagent is stable for months.

2. EDTA solution:
   Dissolve 2 g of disodium ethylene diamine tetra acetate in 1 L of 0 1 N NaOH.
3. Color reagent:
   Dissolve 60 g of ferric nitrate [$Fe_2(No_3)_3$ 9 H_2O] in 500 ml of water, add 15 ml of concentrated nitric acid and add water upto 1 litre
4. Calcium standard, 5 mEq/L:
   Dissolve 125 mg of dry calcium carbonate (dried at 120°C for 30 minutes in a hot-air-oven) in 40 ml of 0.1 N hydrochloric acid and dilute to 500 ml with water.

Procedure·

Label three centrifuge tubes as Test (T), Standard (S) and Blank (B)

Into T pipet 0.2 ml serum and 5 ml calcium reagent and mix.

Into S pipet 0.2 ml calcium standard solution and 5 ml of calcium reagent. Mix

Into B pipet 5 ml of calcium reagent.

Stand for 30 minutes at room temperature with mixing in between centrifuge at 300 rpm for 10 minutes. Carefully invert the tubes, one after another, and pour off the supernatant. Keep them inverted over a pad of filter paper for 5 minutes for draining

Add 1 ml of EDTA solution to each of T, S and B. Mix. Place a marble each over the mouth of tubes. Keep the three tubes in boiling water bath for 10 minutes. Cool. Add 3 ml of color reagent to each of T, S and B. Mix. Read the absorbance at 450 nm.

The procedure is summarised in Table VII-40

Table VII-40

Reagents	*B*	*S*	*T*
Serum	—	—	0 2 ml
Standard calcium	—	0.2 ml	—
Calcium reagent	5 0 ml	5.0 ml	5 0 ml
Mix Stand 30 minutes at room temperature, Centrifuge			
Pour off supernatant			
EDTA solution	1 0 ml	1 0 ml	1 0 ml
Keep in boiling water bath, 10 minutes, Cool			
Color reagent	3 0 ml	3 0 ml	3 0 ml
O.D. (450 nm or Blue filter)			

Calculation:

$$\text{Serum calcium in mEq/L} = \frac{\text{O.D. of T} - \text{O.D. of B}}{\text{O D. of S} - \text{O D. of B}} \times 10$$

To convert the result to mgs% multiply mEq/L by 2.

Note:

1. The glasswares must be perfectly clean. This is done by washing the tubes in chromic acid wash solution
2. Pouring off the supernatant is done carefully not to lose any precipitate.

4.5.3 2 Method· O-Croselphtalein complexene.

Sample. Serum or plasma

Principle.

Calcium in the serum forms a violet colored complex with O-cresolphthalein complexene S-Hydroxyquineline included in the reagent prevents interference by magnesium.

Reagents:

Reagent A.

Transfer 210 g of diethanolamine and 300 g of urea to about 900 ml of double distilled water into a 1 litre beaker. Dissolve the

crystals Adjust the pH to 11 7 with acetic acid. Transfer to 1 litre flask and make upto 1 litre with double distilled water. The reagent is stable at least for one month.

Reagent B:

Dissolve 64 mg of O-cresolphthalein complexene, 1.16 g of 8-Hydroxyguineline and 2.5 ml glacial acetic acid in 250 ml of ethanol.

Add 300 g of urea to this solution. Make up the volume to 1 litre with double distilled water. The reagent is stable for at least one month

Working reagent:

Mix equal volumes of reagent A and reagent B just before use. (Calculate the approximate volume of reagent required and prepare the required volume of reagent for the day).

Standard calcium.

10 mg/100 ml or 5 mEq/L. Dissolve 125 mg of dry calcium carbonate in 40 ml of 0.1 N HCl and dilute to 500 ml with water

Procedure·

Pipet 0.2 ml serum and 1.8 ml water to a test tube. Mix.

Transfer 0 5 ml from this to a test tube marked Test (T)

Add 5 ml of working reagent.

To a second test tube add 0.2 ml of calcium standard solution and 1 8 ml of water.

Transfer 0 5 ml from this to a test tube marked Standard (S). Add 5 ml of working reagent Mix.

Label a 3rd test tube as Blank (B) and add 0.5 ml water and 5 ml of working reagent. Mix.

Read absorbances of B, S and T at 540 nm or using green filter.

Calculation.

$$\text{mgs of calcium per 100 ml serum} = \frac{\text{O.D. of T} - \text{O.D. of B}}{\text{O.D of S} - \text{O.D. of B}} \times 10$$

When mgs% is divided by 2 mEq/L is obtained

Interpretation:

Normal value ranges from 9-10.8 mg per 100 ml serum or 4 5-5.4 mEq/L Calcium level is lowered in tetany, hypoparathyroidism, renal insufficiency, sprue, diarrhoea, childhood rickets etc. Calcium level is raised in primary hyperparathyroidism, Vitamin D overdosage, bone tumours etc.

4.5.4 Determination of Serum Inorganic Phosphorus

Method: Fiske—Subba Row

Specimen·

Serum collected from venous blood. Plasma is also suitable.

Principle·

Serum proteins are precipitated by trichloro acetic acid Molybdic acid added to protein-free filtrate converts phosphate to phosphomolybdate. Alpha-nathol sulfonic acid added reduces phosphomolybdate to a blue colored compound. The intensity of blue color is measured photometrically using red filter or at 660 nm

Reagents:

1. Trichloro acetic acid, 10%
   Dissolve 100 g TCA in water and make upto 1 litre.
2. Molybdate reagent·
   To 200 ml of water add 83 ml sulfuric acid, keeping cold under tap or cold water. Dissolve 25 g of ammonium molybdate in this solution. Dilute this to 1 litre with water. Solution is stable.
3. 1, 2, 4-amino naphthol sulfonic acid reagent or ANSA Reagent.
   Dissolve 0 125 g of 1, 2, 4-amino naphthol sulfonic acid, 7.28 g of sodium metabisulfite and 0.25 g of anhydrous sodium sulfite in 50 ml of water. Filter. Store in a brown bottle. The reagent is stable for one month kept in refrigerator.
4. Standard phosphorus solution 0.08 mg per ml solution:
   Dissolve 0.351 g of dried potassium dihydrogen phosphate in about 500 ml water in a litre flask. Add 8 ml concentrated HCl and dilute to one litre. Prepare fresh once a month.

Procedure:

Test Pipet 2 ml of serum and 8 ml of 10% TCA or 1 ml serum and 9 ml of 10% TCA in a test tube. Mix Stand for a few minutes. Filter through Whatman No 1 filter paper

Transfer 5 ml filtrate to a test tube labeled as T.

Blank: To a test tube labeled as B pipet 1 ml water and 4 ml of 10% TCA.

Standard. To a test tube labeled as S pipet 0.5 ml of standard phosphorus solution, 0.5 ml water and 4 ml of 10% TCA Mix

Add 1 ml of molybdate reagent to each of the three tubes followed by 0 4 ml of ANSA. Mix well Stand for 5 minutes Add 3 6 ml of water to each of B, S and T. Mix

Read optical density of B, S and T using red filter or at 680 nm in a colorimeter.

The steps in the procedure are summarised in Table VII-41

Table VII-41

Reagents	*B*	*S*	*T*
Serum	—	—	2 ml
TCA, 10%	—	—	8 ml
Mix Stand 5 minutes. Filter			
Water	1 ml	0 5 ml	—
Standard phosphorus solution	—	0 5 ml	—
Filtrate	—	—	5 ml
TCA, 10%	4 ml	4 ml	—
Molybdate reagent	1 ml	1 ml	1 ml
ANSA	0 4 ml	0 4 ml	0 4 ml
Mix Stand for 5 minutes			
Water	3.6 ml	3.6 ml	3 6 ml
O.D Reading (Red filter or at 660 nm)			

Calculation

$$\text{mgs phosphorus per 100 ml serum} = \frac{\text{O.D of T} - \text{O D of B}}{\text{O.D. of S} - \text{O.D of B}} \times 0\,04 \times \frac{100}{1}$$

$$= \frac{\text{O D. of T} - \text{O.D. of B}}{\text{O D. of S} - \text{O D. of B}} \times 4$$

Result:

Serum inorganic phosphorus level mgs/100 ml

Note·

1. The determination should be made immediately after withdraw-

ing blood. The inorganic phosphorus increases when blood is allowed to stand

2. Use double distilled water throughout

Interpretation

The phosphorus concentration in serum is inversely proportional to the calcium concentration such that Ca × P = 40

The normal value for adults is 2.5-4.5 mgs % and for children 4-6 mgs %.

It is raised in hypoparathyroidism, renal insufficiency and childhood rickets

It is decreased in hyperparathyroidism, calcium absorption disturbances, Vitamin D deficiency and Ostoomalasia.

Note:

The method is employed to determine phospahte in urine, urine is adjusted to pH 5 by adding 1 N HCl It is diluted 1 in 10 with water. Proceed same as above. Collect 24 hour urine sample

4.6 Diagnostic Enzymes

Enzymes are organic catalysts. All enzymes are proteins. In presence of enzymes the chemical reactions proceed at a very fast rate than in their absence. Enzyme acts only on specific substance specific for that enzyme That substance is called the substrate for that enzyme. In the initial stages the increases in substrate concentration increase that rate of chemical reaction and after a point any increase in substrate concentration will show no change in reaction rate. Enzyme shows maximum activity at a particular temperature specific for the enzyme. That temperature is called the optimum temperature. Likewise the optimum pH is the pH at which enzyme shows maximum activity. An increase or decrease in temperature or pH around its optimum will result in loss of activity and finally usually above 60°C enzyme activity will be at zero. Usually the enzyme activity is arrested by precipitating the enzyme protein using various deproteinising agents. Enzymes are commonly named after adding—ase to their substrates, e.g. urease, lactase, maltase, amylase, etc

There are some enzymes which increase in some diseases thus facilitating the easy and early diagnosis of diseases. Those are called diagnostic enzymes Serum amylase activity increases in pancreatitis, transaminases increase in liver diseases. In cardiac diseases glutamate-oxalocetate transminase, lactate dehydrogenase and creatine phospho-kinase increase.

Expressing the Enzyme Activity

Enzyme concentration is very small compared to its substrate concentration So enzyme activity is expressed in terms of the amount of product liberated.

International Unit

When one micromole of the product is liberated per litre of the sample per minute the enzyme concentration is expressed as one I.U.

4.6.1 Determination of Transminases

4 6 1.1 Determination of Serum Glutamate—Oxaloacetate Transaminase (SGOT or Aspartati Transaminase)

Method Reitman-Frankel

Principle.

The enzyme catalyses the conversion of DL-aspartic acid to oxaloacetate at a temperature of 37°C as per the following reaction.

DL-aspartic acid + alpha-ketoglutaric acid →

oxaloacetic acid + glutamate

The oxaloacetate formed is spontaneously converted to pyruvate which then forms a pink color with 2, 4-dinitrophenyl hydrazine.

Reagents

1 Phosphate buffer 0.1 M, pH 7 4·
Dissolve 13 97 g of dipotassium hydrogen phosphate (K_2HPO_4) and 2 69 g of potassium dihydrogen phosphate (KH_2PO_4) in water and make up to one litre. Adjust the pH to 7.4. Store at 4°C in a refrigerator.

2. Alpha-ketoglutaric acid·
Dissolve 292 mgs of alpha-ketoglutaric acid in a little of water. Adjust the pH to 7.4 with 1 N NaOH and make up the volume to 100 ml. Stored in deep-freezer refrigerator, keeps for 2-3 months.

3 GOT substrate
Weigh out 665 mgs of DL-aspartic acid or 332 5 mgs of L-aspartie acid Add 5 0 ml of 1N NaOH, 2 5 ml of reagent 2 and 17.5 ml of phosphate buffer. Mix well to dissolve Check the pH to 7.4 and make necessary adjustment using 1 N NaOH or phosphoric acid Keeps in freezer for months.

4. Calibration standard:
Dissolve 22 0 mgs sodium pyruvate in 100 ml of phosphate buffer Prepare fresh every month.

5. Color reagent.
Dissolve 19.8 mgs of 2, 4-dinitrophenyl hydrazine in 100 ml of

1 N HCl. Store in amber colored bottle. Stable for months

5. Sodium hydroxide, 1 N
   Prepare 1 N sodium hydroyid by titrating against 1 N oxalic acid solution.

7. Sodium hydroxide 0.4 N.
   Dilute 40 ml of 1 N NaOH to 100 ml with water.

8. Hydrochloric acid 1 N:
   Dilute 45 ml of concentrated HCl to 500 ml. Titrate against 1 N NaOH and adjust to 1 N.

Specimen: Non-Hemolysed serum

Procedure:

Add 0.5 ml of GOT substrate into a test tube labeled Test (T).
Keep in water both at 37°C for 5 minutes
Add 0.1 ml serum and mix, keep the tube in the water bath incubate for exactly 60 minutes.
Add 0.5 ml of color reagent after 60 minutes of incubation.
Take another test tube and label it as control (C).
Add 0.5 ml of GOT substrate, 0.5 ml of color reagent and 0.1 ml of serum Mix.
Let stand both the tubes at room temperature for 20 minutes.
Add 5 ml of 0.4 N NaOH to both tubes. Mix.
After 5 minutes read the absorbance at 505 nm or using green filter.

Calibration standard:

Label six tubes as 1, 2, 3, 4, 5 and 6 Pipet the reagents as given in the Table VII-42.

Table VII-42

Tube No	*Std. Sodium pyruvate*	*GOT substrate*	*Water*
1	0	1.0 ml	0.2 ml
2	0.1 ml	0 9 ml	0 2 ml
3	0 2 ml	0.8 ml	0 2 ml
4	0.3 ml	0.7 ml	0 2 ml
5	0 4 ml	0.6 ml	0.2 ml
6	0 5 ml	0.5 ml	0.2 ml

Add 1 ml color reagent to all tubes.
Let stand at room temperature for 20 minutes.
Add 10 ml of 0.4 N NaOH to all tubes. Mix.
Let stand for 5 minutes.
Read absorbances at 505 nm or using green filter.

Table VII-43

Tube No	*O D.*	*GOT Units/100 ml*
1		0
2		20
3		55
4		95
5		148
6		216

Draw a calibration curve by plotting units per 100 ml on x-axis and O.D. on y-axis. Read the enzyme level in units per 100 ml of test from the graph

Note.

If the SGOT level is about 216 units per 100 ml repeat the test taking the serum diluted 1+9 with physiological saline. The value obtained from the graph is multiplied by 10.

Interpretation:

GOT is found in most of the tissues like heart, liver, muscle, kidney etc. The normal range of this enzyme is upto 35 SF units per 100 ml.

GOT is raised in infective, toxic and viral hepatitis.

In chronic hepatitis and cirrhosis of the liver, GOT is raised moderately. GOT rises in Myocardial infarction. GOT increases after 6 hours of attack and reaches maximum after 12 to 36 hours and returns to normal after 6 days.

In progressive muscular dystrophy also GOT is increased in serum.

4.6.1.2 Determination of Serum Glutamate-Pyruvate Transminase (SGPT or Alanine Transminase)

Method: Reitman-Frankel.

Principle:

This enzyme catalyses the conversion of L-alanine to pyruvate at a temperature of 37°C as per the following reaction

L-alanine + α-ketoglutaric acid → pyruvate + glutamic acid

The reaction is reversible in vivo. The pyruvate liberated will develop pink color on treatment with dinitrophenyl hydrazine The enzyme activity is quantitated using sodium pyruvate calibration standard.

Reagents:

1. Phosphate buffer 0 1 M, pH 7 4
   Dissolve 13.97 g of dipotassium hydrogen phosphate (K_2HPO) and 2.69 g of potassium dihydrogen phosphate (KH_2PO) in water and make upto one litre Adjust the pH to 7 4. Store at 4°C in a refrigerator.

2. Alpha-ketoglutaric acid.
   Dissolve 292 mgs of alpha-ketoglutaric acid in a little of water, adjust the pH to 7 4 with IN NaOH and make up the volume to 100 ml. Store in deep-freezer refrigerator.

3 GPT substrate·
   Weigh 445 mgs of L-alanine Add 5.0 ml of 1 N NaOH slowly with mixing, 2.5 ml of alpha-ketoglutaric acid and 17.5 ml of phosphate buffer. Mix to dissolve. Adjust the pH to 7 4 by adding NaOH or phosphoric acid. Stored in deep-freezer refrigerator, keps for months.

4. Calibration standard.
   Dissolve 22.0 mgs of sodium pyruvate in 100 ml phosphate buffer. Store in deep-freezer. Keeps for one month.

5 Color reagent:
   Dissolve 19.8 mgs of 2, 4-dinitrophenyl hydrazine in 100 ml of 1 N HCl. Stored in amber colored bottle, keeps indefinitely.

6. Sodium Hydroxide, 0.4 N·
   Dissolve 16.0 g of sodium hydroxide in water and dilute to one litre Titrate and standardise to 0.4 N using 0 4 N oxalic acid solution Store in polythene bottle.

7. Sodium Hydroxide, 1 N·
   Dissolve 40 g NaOH in water and make upto one litre. Check the

normality by titration using primary standard 1 N oxalic acid and adjust.

8. Hydrochloric acid 1 N·
   Dilute 45 ml of concentrated HCl to 500 ml with water. Titrate against 1 N NaOH and adjust to 1 N.

Procedure:

Add 0.5 ml of GPT substrate into a test tube labeled T.

Keep in the water bath at 37°C for 5 minutes

Add 0.1 ml serum and mix, keeping inside the waterbath.

Incubate for exactly 30 minutes.

Add 0.5 ml of dinitrophenylphydrazine solution (color reagent) at the end of 30 minutes.

Take a test tube and mark as "C" (control)

Add 0.5 ml GPT substrate, 0.5 ml of color reagent and 0.1 ml of serum. Mix.

Let stand both the tubes at room temperature for 20 minutes.

Add 5 ml of 0.4 N sodium hydroxide to both T and C

After 5 minutes read the absorbances at 505 nm or using green filter against water.

Calibration Curve:

Label six tubes as 1, 2, 3, 4, 5 and 6. Pipet the solutions indicated in the Table VII-44.

Table VII-44

Tube No.	*Std Pyruwate solution*	*(GPT) Substrate*	*Water*	*Optical density*
1	0	0 1 ml	0 2 ml	
2	0.1 ml	0.9 ml	0 2 ml	
3	0 2 ml	0 8 ml	0 2 ml	
4	0 3 ml	0 7 ml	0.2 ml	
5	0 4 ml	0 6 ml	0 2 ml	
6	0.5 ml	0.5 ml	0.2 ml	

Add 1.0 ml of color reagent to all tubes.

Allow to stand for 20 minutes at room temperature.

Add 10 ml 0 4 N sodium hydroxide, wait for 5 minutes.

Read absorbances at 505 nm or using green filter.

The GPT units in standards as given by Sigma-Frankel are as follows:

Draw a calibration curve by taking units on x-axis and O.D. on y-axis.

Table VII-45

Tube No	*O.D*	*GPT in SF units*
1		0
2		23
3		50
4		83
5		125

Note.

1 Calibration curve should be repeated occasionally.

2. Serum keeps for four days without change when kept refrigerated

3. The time of incubation is strictly adhered to

4 In place of L-alanine, DL-alanine can be used, but the weight to be taken is double

Interpretation.

Normal range of SGPT is 35 Sigma-Frankel Units (SF Units/100 ml). The level is increased in liver diseases (Infective hepatitis and cirrhosis)

4.6.2 Determination of Phosphatases

Phosphatases are enzymes which catalyse the splitting off of phosphoric acid from mono-phosphoric acid esters Two types are commonly estimated in serum, alkaline phosphatase with maximum activity at about pH 10 and acid phosphatase with maximum activity at about pH 5

4.6.2.1 Alkaline Phosphatase:

Alkaline phosphatases are present in most tissues the richest sources being bone and the bile canaliculi in the liver. In these sites it seems the enzyme is involved in the transport of phosphate across cell membranes In the determination of serum alkaline phosphatase Kay and Bondansky (1947) used sodium β-glycorophosphate and

determined the inorganic phosphate liberated by the enzyme action King and Armstrong introduced disodium phenyl phosphate with which either phenol or inorganic phosphate liberated can be determined.

Method I

Principle:

Alkaline phosphatase splitts off disodium phenyl phosphate at pH 10 liberating phenol. The liberated phenol reacts with Folin-Ciocalteu reagent in presence of sodium carbonate to develop blue color, the absorbance of which is measured photometrically.

Reagents:

1. Disodium phenyl phosphate, 0 01 M
   Dissolve 1.09 grams of disodium phenyl phosphate in water and make upto 500 ml. Bring quickly to the boil. Cool, add a little chloroform and keep in the refrigerator Keeps at least for a month.

2. Sodium carbonate-sodium bicarbonate buffer, p.1 M, pH 10:
   Dissolve 3.18 grams of anhydrous sodium carbonate and 1.68 gms of sodium bicarbonate in water and make upto 500 ml. Adjust the pH to 10

3 Buffered substrate for use
   Prepare by mixing equal volumes of solutions 1 an 2 This has a pH of 10 Prepare fresh every day.

4 Phenol reagent of Folin and Ciocalteu (1927):
   Dissolve 100 gms of sodium tungstate (Na_2WO_4, $2H_2O$) and 25 gms of sodium molybdate (Na_2MoO_4, $2H_2O$) in about 700 ml of water in a 2-litre round bottomed flask Add 50 ml of syrupy 85 per cent phosphoric acid and 100 ml of concentrated hydrochloric acid. Reflux for ten hours Use all-glass apparatus if possible, otherwise wrap the stopper in tin foil. Add 150 gms of lithium sulphate, 50 ml of water and a few drops of bromine Boil without a condenser for about a quarter of an hour to remove the excess of bromine Cool, make upto a litre, and filter The solution should have no greenish tint. This is an improved phenol reagent which reduces the possibility of turbidity due to insoluble urates This kept in dark and cold keeps indefinitely.

Dilute this solution 1 in 3 for use in this determination. That is, to 1 volume of phenol reagent add 2 volumes of water

5 Twenty per cent sodium carbonate solution·
Dissolve 20 gms of anhydrous sodium carbonate in water and make up to 100 ml. This solution is almost saturated at room temperature and so should be kept in a warm place to prevent the salt from crystallizing out

6. Standard phenol solution—Stock solution One hundred mg of phenol per 100 ml of solution.
Dissolve 1 g of pure crystalline phenol in 0 1 N hydrochloric acid and make upto 1 litre with the acid This can be standardised as follows· place 25 ml into a 250 ml flask. Add 50 ml of 0 1 N sodium hydroxide and heat to 65°C. To the hot solution add 25 ml of 0 1 N iodine. Stopper and allow to stand for thirty to forty minutes. Add 5 ml of concentrated hydrochloric acid and titrate the excess iodine with 0 1 N sodium thiosulphate. Each ml of 0.1 N iodine is equivalent to 1.57 mg of phenol Store at 4°C in refrigerator

7. Diluted phenol standard for use·
Dilute the stock standard 1 in 10 to obtain a standard solution containing 10 mg phenol per 100 ml of solution. Prepare this once a week.

8. Standard phenol solution and reagent, containing 0 5 mg phenol per 100 ml:
Take 2.5 ml of the dilute standard, add 15 ml of the diluted phenol reagent and make upto 50 ml with water. This is best prepared daily.

Technique:

Pipet 6 ml of the buffer-substrate into a test tube and place in the water bath at 37°C for a few minutes. Add 0.3 ml of serum, preferably without removing from the bath. Mix and cork and allow to remain in the bath for exactly 15 minutes. Then remove and immediately add 2.7 ml of the diluted phenol reagent. At the ame time set up a tube for the control containing 6 ml of substrate and 0.3 ml of serum, reagent. Mix well in both cases and centrifuge.

Take 4 ml of supernatant fluid from each and add 1 ml of 20 per cent sodium carbonate. Put up a standard prepared by adding 1 ml of

sodium carbonate solution to 4 ml of the standard phenol and reagent. Place the three tubes in 37°C water both for 15 minutes and read in the colorimeter. As blank take 2 8ml of water and add 1.2ml diluted phenol reagent and 1 ml of 20 per cent sodium carbonate. A red filter is used, with transmission at 680 millimicrons.

Calculation.

The result is expressed in mg of phenol liberated by 100ml of serum in 15 minutes at 37°C each unit corresponding to the liberation of 1 mg of phenol per 100ml of serum:

mg phenol per 100ml unknown

$$= \frac{\text{O.D. of T—O D. of C}}{\text{O.D of S} - \text{O.D. of B}} \times \text{amount of phenol in standard} \times \frac{\text{ml volume after adding phenol reagent}}{\text{ml supernatant taken}} \times \frac{100}{\text{amount of serum used}}$$

$$= \frac{\text{O.D. of T} - \text{O.D. of C}}{\text{O.D. of S} - \text{O.D. of B}} \times 0.2 \times \frac{9}{4} \times \frac{100}{0.3}$$

$$= \frac{\text{O.D. of T} - \text{O.D. of C}}{\text{O.D. of S} - \text{O.D. of B}} \times 15$$

= Units phosphatase per 100 ml serum

A standard curve can be prepared as follows using the diluted phenol standard (7) above (See Table VII-46):

Table VII-46

Mg Phenol/100 ml serum	0	6	12	18	24	30	36
Ml dilute phenol standard	0	0 2	0.4	0.6	0 8	1 0	1 2
Ml. water	7 0	6 8	6 6	6 4	6 2	6 0	5.8

Add 3 0 ml of dilute Folin and Ciocalteu reagent and 2.5 ml of 20% carbonate and proceed as described above.

Read unknown and blank and substract the mg phenol per 100 ml of the latter from that of the former to obtain the units of phosphatase per 100 ml serum

Notes:

1 To convert King and Armstrong units into I.U per litre multiply by 1,000 and divide by 94 (M Wt. of phenol) to convert to micromoles, multiply by 10 to change to per litre, and divide by 15 to make it per minute. Thus.

$$\text{I.U per litre} = \text{KA units} \times \frac{1000}{94} \times \frac{10}{15} = \text{KA units} \times 7.1$$

So the normal range is 22-92 I.U. per litre.

2. With high serum phoisphatase values it is advisable to use less serum, since the reaction ceases to be proportional to the amount of phosphatase present. So, for values of more than 40 units per 100 ml dilute the serum 1 in 5 with 0.9 percent sodium chloride and repeat. Occasionally it may be necessary to dilute even more With such a modification, values of 100 to 200 units are not uncommon, and even higher figures may be obtained, though only rarely.

Method II: King and King (1954)

In the presence of alkaline exidising agents 4-amino-phenazone (4-aminoantipyrine) gives a red or purple colour with phenol, liberted from disodium phenyl phosphate by the enzyme phosphatase. It has the advantage over the earlier technique that proteins do not react and need not be precipitated, thus eliminating one step in the procedure Colour development is rapid and the color is stable for at least an hour

Reagents:

Buffer: (pH 10.14 at 20°C, 9.90 at 37°C). Dissolve 3.18 gms of anhydrous sodium carbonate and 1.68 gms of sodium bicarbonate in water and make upto 500 ml Keep at 4°C The pH should lie within ± units of its nominal values

Substrate (0 01 M): Dissolve 1.09 g of disodium phenyl phosphate, in 500 ml boiled and cooled distilled water and 2 ml chloroform as preservative.

0 4 N sodium hydroxide

0 6 N sodium bicarbonate · 0 41 gms of anhydrous sodium carbonate/litre.

4-amine antipyrine: 6 gm/litre. Filter and store in brown bottle

Potassium ferricyanide. 4 gm/litre. Store in brown bottle. If a slight green tinge appears, prepare fresh reagent.

Standard Phenol 1 mg/ml: 100 mgs of pure phenol in 100 ml of 0.1 N HCl. Keep at 4°C in a brown bottle. It lasts at least for one month.

Working standard: 0.05 mg/ml—Dilute 5 ml of stock phenol standard to 100 ml with distilled water; preserve with a few drops of chloroform and keep at 4°C in a brown bottle. Lasts for one week

Procedure

Mix 1 ml of buffer with 1 ml substrate in a test tube and place in a water bath at 37°C ± 0 02°C for 3 minutes Add 0 1 ml of serum, mix gently and incubate for exactly 15 minutes. Stop reaction by the addition of 1 ml of 0.4 N NaOH.

Control: In a test tube mix 1 ml buffer with 1 ml substrate and 1 ml 0.4 N NaOH followed by 0.1 ml serum.

Standard: In a test tube mix 1 ml buffer with 0.5 ml std. (standard) (0.05 mg/ml) and 0 5 ml distilled water, 1 ml 0.4 N NaOH

Blank: Mix 1.0 ml buffer, 1 ml water and 1.0 ml 0 4 N NaOH

To each tube add 1 ml of 0.6 N sodium bicarbonate followed by 1 ml of 4-amino antipyrine solution and 1 ml of potassium ferricyanide solution, mixing each tube well after each addition With the successive addition adjust the pH and develop the colour, failure to mix thoroughly leads to irregular results because colour development is marked by pH dependent.

Compare the reddish brown colors immediately at green filter (510 nm) avoiding, exposure to strong sunlight.

Calculation ·

The amount of phenol present in the standard tube is 25 microgram. Then the phenol produced in 15 minutes in the tube is

$$\frac{T-C}{S-B} \times 25 \text{ microgram}$$

Hence 100 ml serum would liberate,

$$\frac{T-C}{S-B} \times 25 \text{ mgs of phenol}$$

Since 1 King Armstrong Unit is the production of 1 mg of phenol in 15 minutes under the conditions of the test

$$\text{Serum alkaline phosphate in K.A Units/100 ml} = \frac{T-C}{S-B} \times 25$$

Interpretation

Alkaline phosphatase is formed in most organs being plentiful in the small intestines, bones, liver and kidneys The normal ranges is between 3-13 K A Units per 100 ml or 23-92 I.U per litre, being higher in children upto 20 units In bone diseases serum alkaline phosphatase increases when bone regeneration is taking place There is marked increase in rickets, parallel to the severity of disease. In osteomalacia, i e , adult rickets also an increase is seen.

In the steatorrhoeas the impaired absorption of Vitamin D and of calcium leads to bone changes and to moderate increase in alkaline phosphatase Still phosphatase estimation alone is of limited value In bone disease for differential diagnosis it is necessary to take the findings of serum such as calcium, inorganic phosphorus and proteins

Serum alkaline phosphatase increases in jaundice in both infective and post-hepatic obstructive jaundice. However the rise is usually much greater in obstructive jaundice than in jaundice due to hepatitis

4.6.2.2 Acid Phosphatase

Method: Gutman and Gutman (1938-1940)

The King-Armstrong method for alkaline phosphatase was adapted for acid phosphatase by Gutman and Gutman, which substituted a different buffer so that the reaction could be carried out at pH 4.9.

Reagents:

Same as for alkaline phosphatase except buffer.

1. Citric acid—sodium citrate buffer 0.1 M pH 4 9·
   Dissolve 21 gms of crystaline citric acid in water and add 188 ml of 1N NaOH (40 g of sodium hydroxide in one litre solution in water) and make up to 500 ml with water Adjust to pH 4 9 by adding normal NaOH or HCl.

0.4 N sodium hydroxide

0 6 N sodium bicarbonate: 0.41 gms of anhydrous sodium carbonate/litre.

4-amine antipyrine: 6 gm/litre. Filter and store in brown bottle.

Potassium ferricyanide: 4 gm/litre. Store in brown bottle If a slight green tinge appears, prepare fresh reagent.

Standard Phenol 1 mg/ml: 100 mgs of pure phenol in 100 ml of 0.1 N HCl. Keep at 4°C in a brown bottle. It lasts at least for one month.

Working standard: 0.05 mg/ml—Dilute 5 ml of stock phenol standard to 100 ml with distilled water, preserve with a few drops of chloroform and keep at 4°C in a brown bottle. Lasts for one week.

Procedure

Mix 1 ml of buffer with 1 ml substrate in a test tube and place in a water bath at 37°C ± 0 02°C for 3 minutes Add 0 1 ml of serum, mix gently and incubate for exactly 15 minutes Stop reaction by the addition of 1 ml of 0.4 N NaOH.

Control: In a test tube mix 1 ml buffer with 1 ml substrate and 1 ml 0.4 N NaOH followed by 0.1 ml serum.

Standard: In a test tube mix 1 ml buffer with 0.5 ml std. (standard) (0.05 mg/ml) and 0.5 ml distilled water, 1 ml 0.4 N NaOH

Blank. Mix 1.0 ml buffer, 1 ml water and 1.0 ml 0 4 N NaOH.

To each tube add 1 ml of 0.6 N sodium bicarbonate followed by 1 ml of 4-amino antipyrine solution and 1 ml of potassium ferricyanide solution, mixing each tube well after each addition With the successive addition adjust the pH and develop the colour, failure to mix thoroughly leads to irregular results because colour development is marked by pH dependent.

Compare the reddish brown colors immediately at green filter (510 nm) avoiding, exposure to strong sunlight.

Calculation

The amount of phenol present in the standard tube is 25 microgram. Then the phenol produced in 15 minutes in the tube is

$$\frac{T - C}{S - B} \times 25 \text{ microgram}$$

Hence 100 ml serum would liberate,

$$\frac{T - C}{S - B} \times 25 \text{ mgs of phenol}$$

Since 1 King Armstrong Unit is the production of 1 mg of phenol in 15 minutes under the conditions of the test

$$\text{Serum alkaline phosphate in K A. Units/ 100 ml} = \frac{T-C}{S-B} \times 25$$

Interpretation

Alkaline phosphatase is formed in most organs being plentiful in the small intestines, bones, liver and kidneys The normal ranges is between 3-13 K.A Units per 100 ml or 23-92 I.U per litre, being higher in children upto 20 units In bone diseases serum alkaline phosphatase increases when bone regeneration is taking place There is marked increase in rickets, parallel to the severity of disease. In osteomalacia, i.e., adult rickets also an increase is seen

In the steatorrhoeas the impaired absorption of Vitamin D and of calcium leads to bone changes and to moderate increase in alkaline phosphatase. Still phosphatase estimation alone is of limited value In bone disease for differential diagnosis it is necessary to take the findings of serum such as calcium, inorganic phosphorus and proteins

Serum alkaline phosphatase increases in jaundice in both infective and post-hepatic obstructive jaundice. However the rise is usually much greater in obstructive jaundice than in jaundice due to hepatitis

4.6.2.2 Acid Phosphatase

Method: Gutman and Gutman (1938-1940)

The King-Armstrong method for alkaline phosphatase was adapted for acid phosphatase by Gutman and Gutman, which substituted a different buffer so that the reaction could be carried out at pH 4.9.

Reagents:

Same as for alkaline phosphatase except buffer.

1. Citric acid—sodium citrate buffer 0.1 M pH 4.9.
   Dissolve 21 gms of crystaline citric acid in water and add 188 ml of 1N NaOH (40 g of sodium hydroxide in one litre solution in water) and make up to 500 ml with water Adjust to pH 4.9 by adding normal NaOH or HCl.

Procedure:

Pipet 2 ml of buffer and 2 ml of substrate in two centrifuge tubes, place in the water bath at 37°C for 5 minutes. Add 0.2 ml of serum to the 'test' and mix Incubate at 37°C for one hour. Subsequent procedure is identical with that of alkaline phosphatase.

Calculation:

Same as given for alkaline phosphatase.

Note:

1. Serum for the estimation should be separated without hemolysis and kept frozen in the refrigerator if the estimation can not be carried on the day the blood was taken which is preferable. Prostatic acid phosphatase is unstable and becomes inactivated if the serum is kept at 37°C for one hour.
2. As with the alkaline phosphatase, if high values are obtained the estimation should be repeated using diluted serum.

Interpretation:

Increases in prostatic cancer.

4.6.3 Determination of Serum Amylase

Method: Colorimetric method of Kaplan

Sample: Non-hemolysed serum.

Principle.

Iodine forms a blue color complex with starch. Amylase is the enzyme which splits off starch. Serum is incubated with starch substrate at 37°C for 10 minutes. The enzyme reaction is stopped by the addition of Iodine-EDTA solution. The remaining starch forms a blue color with iodine from which is calculated the amylase activity

Reagents:

1. Nitric acid 1 mol/L:
   Dilute 61 ml of concentrated HNO_3 to one litre with water.
2. Buffered starch solution:

Make a paste of 125 mgs of starch with about 3 ml of water in a 250 ml beaker. Add about 100 ml of water Add 2.435 g of tris (hydroxymethyl) amino mathane and 2.875 g of sodium chloride, AR grade. Bring to boil while stirring. Cool to room temperature. Add 250 mgs of sodium azide as preservative Adjust the pH to 7.4 by the addition of 1 M HNO_3. Transfer to a 250 ml volumetric flask and make up to mark.

3 Stock Iodine—EDTA solution:
Dissolve 12 g of potassium iodide (KI) in 125 ml of water taken in a 500 ml volumetric flask. Add 185 mgs of disodium EDTA and stir until dissolved. Add 5.2 g of iodide and stir until dissolved Make upto 500 ml with water. Store in a brown bottle.

4. Working Iodine—EDTA:
Dilute the stock solution 1 in 100 with water every day.

Procedure:

Label three test tubes as Test (T) Control (C) and Blank (B)

Add 2 ml of buffered starch solution to T, C and B.

Incubate the three tubes at 37°C for 5 minutes Pipet 0.05 ml of serum to test tube T only. Incubate for 10 minutes

Add 15 mlof Iodine-EDTA working solution to test tubes T, C and B. To test tube C add 0.05 ml of serum. To test tube B add 0.05 ml of water. Mix all the tubes thoroughly. Let stand for 3 minutes

Read the absorbance at 640 nm (red filter) after setting to zero with water.

The procedure is summarised in Table VII-47.

Table VII-47

Reagents	*T*	*C*	*B*
Buffered starch solution	2 ml	2 ml	2 ml
Incubate at 37°C for 5 minutes			
Serum	0 05 ml	—	—
Incubate at 37°C for 10 minutes			
Working Iodine-EDTA solution	15 ml	15 ml	15 ml
Serum	—	0 05 ml	—
Water	—	—	0.05 ml
Stand for 3 minutes			
O.D at 640 nm (Red filter)			

Calculation:

Amylase activity in Units/100 ml

$$= \frac{\text{O.D. of control (C)} - \text{O.D. of Test (T)}}{\text{O.D. of control (C)}} \times 900$$

Note:

If the amylase activity is above 1000, repeat the test taking 0.025 ml of serum.

Interpretation:

Normal range is between 80 and 320 units. It is elecated in pancreatitis and mumps.

4.7 Pigments

4.7.1 Determination of Serum Total & Direct Bilirubin

Method: Malloy and Evelyn.

Sample: Serum.

Principle:

Van den Berg developed a colorimetric method for the estimation of bilirubin. Malloy and Evelyn later modified the method.

Here bilirubin is converted to purple color azo-bilirubin on treatment with diazotised sulfanilic acid

Total bilirubin is estimated in methanolic solution. Whereas in the determination of direct bilirubin methanol is substituted by water.

Reagents:

1. Methanol, AR grade.
2. Hydrochloric acid, 1.5%:
   Dilute 15 ml of concentrated HCl to 1 litre with water.
3. Solution A:
   To 985 ml of water add 15 ml of concentrated HCl. Add 1 g sulfanilic acid and dissolve.
4. Solution B.
   Dissolve 0.5 g of sodium nitrite ($NaNO_2$) in water and make upto 100 ml. The solution is stable when kept in refrigerator.
5. Diazo reagent:
   The solution is very unstable, so prepare fresh for every batch of samples. To prepare this add 0.3 ml of solution B to 100 ml of solution A and mix.
6. Standard bilirubin solution, 0.1 mg per ml.:

Weigh 10 mg of bilirubin and dissolve in 100 ml of methanol (AR grade). Store in brown bottle in refrigerator. Stable at least for one month.

Procedure:

Dilute 1 ml of serum to 20 ml with water in a conical flask and mix.

Total Bilirubin:

Label two tubes as Test (T_T) and Blank (T_B)

Into T_B pipet 5 ml of methanol, 1 ml of 1 5% HCl and 4 ml of diluted serum (1 in 20).

Into T_T pipet about 5 ml of methanol, 1 ml of diazo reagent and 4 ml of diluted serum

Mix both the tubes

Stand at room temperature for 30 minutes.

After 30 minutes read absorbance using green filter or at 540 nm.

Direct Bilirubin:

Mark two test tubes as Test (T_D) and Blank (D_B).

Into D_B pipet 5 ml of water, 1 ml of 1.5% HCl and 4 ml of diluted serum

Into T_D pipet 5 ml of water, 1 ml of diazo reagent and 4 ml of diluted serum.

Mix both the tubes.

Let stand at room temperature for 30 minutes.

Read absorbances at 540 nm or using green filter.

Standard:

Label two test tubes as Standard (S) and Blank (S_B).

Into S pipet 9.8 ml of methanol, 1 ml of diazo reagent and 0.2 ml of standard.

In to SB pipet 9.8 ml of methanol, 1 ml of 1.5% HCl and 0.2 ml of standard.

Mix both the tubes.

Stand at room temperature for 30 minutes.

Read absorbance at 540 nm or using green filter.

The method is summarised in Table VII-48.

Table VII-48

Reagents	T_T	T_B	T_D	D_B	S	S_B
Methanol	5 ml	5 ml	—	—	9.8 ml	9 8 ml
Water	—	—	5 ml	5 ml	—	—
Diazo reagent	1 ml	—	1 ml	—	1 ml	—
1 5% HCl	—	1 ml	—	1 ml	—	1 ml
Diluted serum	4 ml	4 ml	4 ml	4 ml	—	—
Standard Bilirubin	—	—	—	—	0 2 ml	0 2 ml
Mix Stand 20-30 minutes						
O D at 540 nm (Green)						

Calculation·

mgs of Total Bilirubin per 100 ml serum

$$= \frac{\text{O.D. of } T_T - \text{O.D of } T_B}{\text{O.D. of S} - \text{O.D. of } S_B} \times 0.2 \times \frac{100}{1.2}$$

mgs of Direct Bilirubin per 100 ml serum

$$= \frac{\text{O.D. of } T_D - \text{O.D of } D_B}{\text{O.D. of S} - \text{O.D os } S_B} \times 10$$

Note:

1. Add serum after the addition of all other reagents otherwise sometimes turbidity may be formed.
2. Bilirubin deteriorates in light. Determination, therefore, is made as soon as possible after the collection of sample. If it is not possible store the serum in dark and in refrigerator. Deterioration of bilirubin still takes place but slower

Interpretation:

Direct bilirubin is water soluble conjugated bilirubin Free bilirubin or water insoluble bilirubin (but soluble in methanol) is unconjugated bilirubin. Bilirubin is formed from hemoglobin by mature RBCs. Upto 1 mg total bilirubin per 100 ml serum is normal.

A raised indirect bilirubin (difference of Total and Direct bilirubin) is found in hemolytic jaundice, jaundice of the newborn, hemolytic anemia, and pernicious anemia. A raised direct bilirubin is found in obstructive jaundice, intrahepatic cholestasis, acute hepatitis etc

4.8 Lipids

4.8.1 Determination of Serum Cholesterol

Method. Zak's Ferric chloride method

Specimen. Serum.

Principle.

Cholesterol in acetic acid reacts with ferric chloride and sulfuric acid to produce a red color. The absorbance of red colored solution is read in a colorimeter.

Reagents.

Use all AR grade chemicals

1. Acetic acid, glacial.
2. Ferric chloride, 0.05% reagent·
   Dissolve 500mgs of ferric chloride, $FeCl_3$ $6H_2O$ in one litre glacial acetic acid Store in brown bottle. It is stable for months
3. Sulfuric acid, AR grade·
4. Stock cholesterol standard solution:
   100 mg per 100 ml in acetic acid Dissolve exactly 100 mgs cholesterol in 100 ml glacial acetic acid Keep in a cool, dark place. Reagent is stable for one month.
5. Working standard cholesterol solution 0.04 mg per ml·
   Dilute 4 ml of stock standard cholesterol solution to 100 ml with ferric chloride reagent (reagent 2). Keep in cool, dark place.

Procedure:

Into a glass-stoppered centrifuge tube transfer 10 ml of ferric chloride reagent

Add 0.1 ml serum using Ostwald pipet

Mix well. Stand for 10 minutes.

Centrifuge at 2000 rpm for 5 minutes. Into 50 ml conical flask or boiling test tube, labeled as T. transfer 5 ml supernatant from above.

In another centrifuge tube add 10 ml of working standard cholesterol and 0 1 ml of 0 9% sodium chloride.

Transfer 5 ml from this to another 50 ml conical flask (or boiling test tube) labeled as S

To a third 50 ml conical flask add 5 ml of ferric chloride reagent

Add 3 ml of concentrated sulfuric acid (AR grade) to each of B, S and T from a burette.

Mix very well. Stand for 20-30 minutes

Set the colorimeter to zero with blank using yellow filter or at 560 nm.

Take absorbance readings of S and T

The procedure is tabulated in Table VII-49.

Table VII-49

Reagents	*B*	*S*	*T*
Ferric chloride reagent	—	—	10.0 ml
Serum	—	—	0.1 ml
Working standard cholesterol	—	10 ml	—
Sodium chloride, 0 9%	—	0.1 ml	—
Mix. Stand for 10 minutes. Centrifuge			
Supernatant	—	5 ml	5 ml
Ferric chloride reagent	5 ml	—	—
Sulfuric acid	3 ml	3 ml	3 ml
Mix Stand for 30 minutes			
O.D (Yellow filter or at 560 nm)			

Calculation:

$$\text{mgs cholesterol per 100 ml} = \frac{T}{S} \times 400$$

Note:

1. The glasswares must be very clean and dry.
2. Transfer concentrated sulfuric acid from a burette.

3. Use "Acetic acid for cholesterol determination" AR grade only

Signficance:

Normal serum cholesterol ranges from 150-240mg per 100ml serum. It may be around 300mg per 100ml in middle age and in pregnancy.

The total serum cholesterol is raised in essential hypertension, nephrotic syndrome, diabetes mellitus, myxoedem and obstructive jaundice.

In atherosclerosis cholesterol level may be raised The risk of cardiac infaraction is higher in such patients

The serum cholesterol level is depressed in hyperthyroidism, severe hepatic damage (cirrhosis) and in malnutrition.

4.9 Liver Function Test

The tests included under liver function tests facilitate the clinician to diagnose and assess the functional capacity of the liver. These are called "Liver Function Test". The tests included are.

1. Total Proteins and Albumin-Globulin Ratio in Serum.
2. Total Cholesterol in Serum
3. Serum Bilirubin (Total and Direct).
4. Serum GOT and GPT.
5. Alkaline Phosphatase
6 Zinc Sulfate Turbidity Test.

The student is already conversant with these tests now, and the methodology of individual tests is given earlier.

4.10 Kidney Function Test

A number of tests are prescribed to assess the functional capacity of kidneys. The following tests adequately help the clinician to get the clear perspective of the kidney function These tests are called "Kidney Function Tests".

The tests included under kidney function test are.

1. Urea clearance test
2 Creatinine clearance test.

The methodology of the individual test is dealt earlier.

PART V
DIAGNOSTIC TESTS ON OTHER BODY FLUIDS

5.1 Gastric Juice

Gastric juice is highly acidic secretion (pH 1.0-2.0) from parietal cells and chief cells. The daily output varies from 2-4 litres. The important constituents of gastric juice are HCl, pepsin and mucin. The abnormal constituents include lactic acid, blood, bile salt and bile pigment.

Table VII-50 Important Constituents of Gastric Juice

1	Hydrochloric acid	HCl in gastric juice provides the optimum pH(1-2) for pepsin action and is responsible for the formation of pepsin from pepsinogen It also acts as an antiseptic or germicide
2	Pepsin	Pepsin is a proteolytic enzyme. It converts proteins like casein to peptones and smaller peptides
3.	Bile (Bile salt and Bile pigment)	The gastric juice is contaminated with bile mainly due to regurgitation The colour of gastric juice will be yellow or green under these conditions
4.	Blood	Blood may be present in gastric juice as a result of injury caused by the introduction of stomach tube (frank or red blood) or due to an ulcer or cancer in the stomach (occult or hidden blood) Under the latter conditions gastric juice may have a dark brown colour
5	Starch	Dietary starch leaves the stomach within 2-3 hours Detection of starch in the post absorptive period indicates a delay in emptying the stomach contents
6.	Lactic acid	Not normally found. Formed during fermentation when the acidity is low.

5.1.1. Analysis of Gastric Juice

Reagents:

1. Topfer's indicator (0 5% ethanol).
2. Gunzberg's reagent.
   Mix 2 parts of 10% phloroglucinol in alcohol with 1 part of 10% vanillin in alcohol.
3. Casein solution
   To 1 gm casein add 20 ml of 0.05 N NaOH. Mix. Add 80 ml hot water (60°C). Agitate until casein dissolves. Pour this solution into a beaker containing 100 ml of 0.1 N HCl with mixing. A prefectly clear solution with a pH of 1 4 is obtained
4. Benzidine solution·
   12% in glacial acetic acid
5. Feuchet's reagent
   Mix 10 ml of 10% $FeCl_3$ with 100 ml of 25% trichloroscetic acid
6. 10% Sodium acetate·
7. Sulphur powder.
8. Hydrogen peroxide.
9. Iodine solution.
   25 gm KI and 12 7 gm iodine in 1 litre.

Tests:

Table VII-51 Tests for Analysis of Gastric Juice

Constituents	*Test detail*	*Observation*
1 HCl	*Test with Topfer's indicator* Add 2 drops of Topfer's indicator to 2 ml gastric juice	Topfer's indicator gives red colour at this low pH. No red colour indicates absence of HCl
	Gunzberg's test Evapoiate carefully a few drops of Gunzberg's reagent in a china dish over a low flame. Streak the hot residue with a glass rod dipped in gastric juice	Note the formation of bright red colour

2. Pepsin	Pipette 5 ml casein and 1 ml gastric juice into a test tube marked A. Mix Into a second test tube B take 5 ml casein and 1 ml gastric juice that has been boiled for 1-2 minutes This serves as control Incubate at room temperature for 15-30 minutes Add sodium acetate drop by drop to the second tube with mixing till maximum precipitation occurs Add the same amount of sodium acetate to the first tube. Compare the amount of precipitates in the tubes.	The first test tube will have very little precipitate indicating the digestion of casein by pepsin
3 Bile sdalt	*Hay's test* To 2 ml gestric juice, sprinkle fine sulphur powder Observe without mixing	Bile salt decreases surface tension Sulphur sinks to the bottom
4. Bilirubin	*Fouchet's test* To 10 ml gastric juice add 5 ml $BaCl_2$ and a pinch of $MgSO_4$. Mix well $BaSO_4$ is precipitate. After 5 minutes filter the solution Unfold the filter paper over 1 or 2 dry filter paper and add few drops of Fouchet's reagent	Wet barium sulphate adsorbs the yellow bilirubin Fouchet's reagent oxidises bilirubin to green biliverdin
5. Blood	*Benzidine test* To 3 drops benzidine solution add 2 drops H_2O_2 Add 1 drop of this mixture to 2 ml gastric juice	A blue or green colour is formed (Unstable).
6. Starch	*Iodine reaction* To 2 ml gastric juice add few drops of iodine solution.	Note the blue colour.

5.1.2 Determination of Free and Total Acidity in Gastric Juice

Reagents:

1. Topfer's indicator (0.5% solution in 50% ethanol).
2 Phenolphthalein (0 1% solution in ethanol)
3. 0 1 N NaOH.

HCl contributes to the free acidity of gastric juice. The acids that contribute to the combined acidity include protein hydrochlorides,

acid phosphate ($H_2PO_4^-$) and organic acids like lactic acid. The organic acids arise due to fermentation in the stomach when acidity is low.

During titration with alkali, HCl is almost completely neutralized at pH 3.5. Topfer's indicator which changes colour at this pH is used for titrating the free acidity. Weak acids on the other hand are neutralized completely above pH 7.0. They can be estimated together with HCl when phenolphthalein is used as indicator in the titration. The difference in the titre values gives a measure of the combined acidity.

Procedure:

Pipette 10 ml gastric juice into a 100 ml conical flask.

Add two drops of Topfer's indicator.

Titrate with NaOH until the red colour changes to orange yellow. Record the titre value.

Add two drops of phenolphthalein and continue the titration until a pale pink colour appears. Note the second titre value

Calculate the volume of 0.1 N NaOH needed to neutralize the free acidity, total acidity and combined acidity in 100 ml gastric juice. One clinical unit of acidity is equal to one ml of 0.1 N NaOH. Acidity is also expressed in milliequivalent per litre.

Table VII-52

Trial No	*Initial reading of the burette*	*Final reading of the burette*	*Volume of alkali required for*	
			Free acidity	Total acidity
1	0	3*	3	—
	0	5**	—	5

*Titre value corresponding to free acidity titration

**Titre value corresponding to total acidity titration

Clinical significance:

The normal values of free acidity and total acidity in fasting sample are respectively 0-30 units/100 ml(0-30 mEq/litre) and 10-50 units/100 ml.

In achlorhydria there will be no secretion of HCl. Achlorhydria

is found in some normal people and the incidence increases with age. When both HCl and pepsin are not secreted the condition is known as achylia gastrica.

Hypochlorhydria (low levels of HCl) is found in pernicious anaemia, gastric carcinoma and chronic gastritis These conditions may ultimately lead to achlorhydria.

Hyperchlorhydria (high levels of HCl) is observed in more than 50% cases of gastric ulcer and 70% cases of duodenal ulcer.

5.1.3 Chemical Investigation of Gastric Function

Chemical examination of the gastric contents yields valuable information in regard to the secretory and motor activities of the stomach and also the nature of some pathological conditions. To obtain a complete picture of the state of the gastric function, the stomach contents should be examined both during the interdigestive period and during the period of gastric stimulation Fractional test meal (F.T.M.) studies and augmented histamine test are two procedures commonly employed to evaluate gastric function.

5 1 3.1 Functional test meal:

Procedure·

The test is done in the morning on the subject who has abstained from food or drink for the previous 12 hours

The contents of the stomach are withdrawn completely using Ryle's tube and the volume is noted. The sample is known as fasting gastric juice.

One point of the test meal (500 ml gruel prepared from 2 spoons of oat meal) is given and the time is noted

Samples of gastric contents are aspirated every 15 minutes for a period of 120-150 minutes.

The free and total acidities of the samples are estimated and a graph is plotted with acidity levels against time (see VII-19).

Qualitative tests are performed with the samples for starch, blood, bile salt, bile pigment, and lactic acid. Make a report.

Value of fasting gastric juice:

The volume in normals is about 20-50 ml Increase in volume may be due to hypersecretion, retention (due to pyloric obstruction) or regurgitation from the duodonum. The volume can be as high as

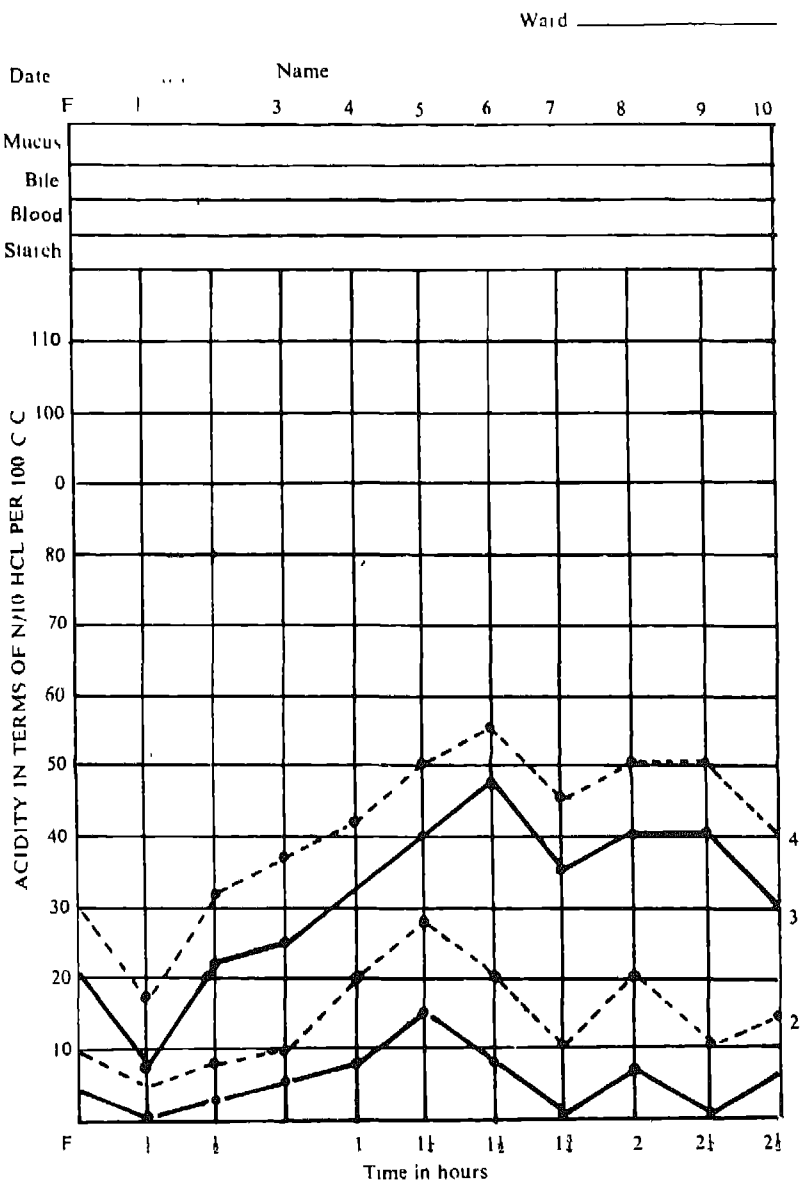


Fig VII-19 FRACTIONAL GASTRIC ANALYSIS

500-1000 ml in pyloric stenosis. Retention can be assessed by giving a couple of charcoal biscuits to the subject 12 hours before the test. If charcoal is found it indicates abnormal retention. Testing for starch in the fasting sample can also be useful in detecting retention.

Acidity·

Normally, the free acidity of fasting sample is in the range 0-30 units. During the test, the free acidity values dip initially and then steadily increase reaching a maximum at 60-90 minutes (about 35-45 units). Thereafter the values return to the basal level in about 120-150 minutes. The total acidity follows a similar pattern, the values being 8-12 units higher than the free acidity values

Higher free acidity values (hyperchlorhydria) in fasting samples are encountered in different ulcers (usually 40-60 units). In most cases of duodenal ulcer as in some cases of gastric ulcer the peak values will rise as high as 100 units and will remain constant during the remaining duration of the test. Achlorhydria and hypochlorhydria are observed in wasting and febrile diseases, microcytic anaemia, chronic gastritis and in many cases of carcinoma of the stomach The combined acidity generally increases in these conditions (usually 30-80 units) especially when there is obstruction. Lectic acid and butyric acid are the major contributors to increase in combined acidity in these cases. They arise due to bacterial fermentation in the stomach which does not take place normally.

Achylia gastrica is found in pernicious anaemia. Sometimes achlorhydria is due to neutralization of HCl caused by regurgitation of pancreatic and bile secretions. This false achlorhydria can be differentiated by augmented histamine test

5.1.3.2 Augmented histamine test:

Histamine is a powerful stimulant for HCl Secretion in the stomach. In the augmented test a higher dose of histamine is given compared to low levels given in the normal histamine test.

Procedure:

After an overnight fast, the fasting gastric content is aspirated (say at 7.00 am).

Gastric contents are collected for 1 hour (from 7-8 am, Basal secretion).

Half-way through this period (at 7 30 am) an intramuscular injection of an anthihistamine drug like mepyramine maleate (50-100 mg) is given to prevent any side effect of histamine.

Histamine is then administered at 8.00 am subcutaneously (0.04 mg histamine/kg body weight).

Gastric contents are aspirated for one hour (post histamine secretion). The free acidity is measured.

In normals the rate of acid secretion can increase about 5-10 fold over the basal level (2.0 ± 2 0 meq. HCl/hr) on histamine administration. The test is very useful in assessing active parietal cell mass. In pernicious anaemia there is a lack of response to histamine. The test is a guideline to the surgeon to limit acid secretion by Vagotomy.

5.2 Cverbrospinal Fluid (CSF)

Apart from blood and urine cerebrospinal fluid is very commonly sent for chemical examination Almost all constituents present in blood are also present in CSF but only a few of these determinations help clinician to diagnose diseases Of these total protein, chloride and sugar are the commonest determinations usually requested by the physician.

The CSF is collected by the physician in the ward by lumber puncture. The procedure of collection of CSF is not simple for the physician or the patient. That being the case the technician should be very careful, because repetition may be impossible in most cases due to limited amount of sample. In instances of limited amounts of CSF sent for analysis, it is advisable to take smaller amounts of sample for individual tests and apply dilution factor in the calculation.

Manually it is important to handle CSF carefully as the sample may contain pathogenic bacteria.It is achieved by cleaning the pipets after use in some bacterial agent or in chromic acid before cleaning and reuse.

5.2.1 Determination of CSF Sugar

Method·

O-Toluidine or Folin-wu method. (The method is same as for glucose estimation in Blood)

Sample: Cerebrospinal fluid.

The proteins are precipitated, immediately after receiving the sample, otherwise bacteria present may utilize glucose. If this is not possible add a little of sodium chloride and refrigerate.

Principle:

Glucose when heated with O-toluidine in acetic acid, forms a

blue-green colored compound, which is measured photometrically.

Reagents·

1. O-Toluidine reagent
   To 5.0 g thiourea (AR grade) add 90 ml O-toluidine and dilute to 1 litre with glacial acetic acid Store in brown bottle. At refrigerator temperature the reagent is stable for a year
2. Trichloroacetic acid 10%.
   Dissolve 10 g of TCA in water and make up the volume to 100 ml.
3. Glucose standard.
   Prepare 100 mgs/100 ml glucose solution in saturated benzoic acid as given under Folin's method

Procedure·

Blank· To 1 4 ml water add 0.6 ml 10% TCA Mix well Transfer 1 ml to a test tube labelled B

Test: To 1 2 ml water add 0 2 ml CSF and mix. Add 0.6 ml 10% TCA. Mix well.

Wait for 5 minutes. Centrifuge for 10 minutes

Transfer 1 ml clean supernatant to a test tube labelled T

Standard: To 1 2 ml water add 0 2 ml glucose standard solution followed by 0.6 ml 10% TCA. Mix well.

To all the three tubes add 5 ml each of O-toluidine reagent and mix

Keep them in boiling water bath for exactly 10 minutes, Cool Read optical densities of B, S and T using red filter or at 630 nm.

The method is summarized in Table VII-53.

Table VII-53

Reagents	*B*	*S*	*T*
Distilled water	1.4 ml	1.2 ml	1.2 ml
Standard glucose solution	—	0 2 ml	—
Cerobrospinal fluid (CSF)	—	—	0.2 ml
TCA, 10%	0.6 ml	0 6 ml	0.6 ml
Mix Wait for 5 minutes. Centrifuge			
Supernatant	1 0 ml	1 0 ml	1.0 ml
O-toluidine	5.0 ml	5.0 ml	5.0 ml
Keep in boiling water for 10 minutes.			
O.D. (Red filter or 630 nm)			

Calculation:

$$\text{mgs per 100 ml CSF} = \frac{\text{O D. of T} - \text{O.D. of B}}{\text{O D. of S} - \text{O.D. of B}} \times 100$$

Note:

1. O-Toluidine is highly corrosive. Handle carefully or preferably use gloves.
2. Use automatic dispensors for O-toluidine reagent. Do not pipet through mouth.
3. The method is directly applied to cerebrospinal fluid and urine.
4. Read the absorbance immediately.

Interpretation:

Normal range is 50-80 mgs per 100 ml of CSF. CSF sugar level is decreased in menigitis.

Small increase are seen in encephalitis, poliomyelitis and cerebral abscess.

5.2.2 Determination of CSF Protein

5.2.2.1 Method. I. Meulemans

Sample: Cerebrospinal fluid.

Principle:

Trichloroacetic acid at 3% concentration produce turbidity with proteins in CSF which is compared with the standard in a colorimeter.

Reagent:

1. Trichloroacetic acid 3% (w/v).
   Dissolve 30 g of TCA in water and dilute to one litre.
2. Stock protein standard—5 mg/ml:
   Dissolve 595 25 mgs of bovine albumin (whose nitrogen content is determined by Kjeldahl method) and100 mgsof sodium azide in water and make upto 100 ml. Store at 4°C in refrigerator.
3. Working standard protein solution, 50 mg per 100 ml:

Dilute 10 ml of the stock standard to 100 ml with 0.9%sodium chloride solution. Store in deep-freezer refrigerator. Prepare fresh every week.

Procedure

Label a test tube as Test T.
Add 1 ml of CSF and 4 ml of 3% TCA. Mix
Label a second test tube as Standard S
Add 1 ml of working standard and 4 ml of 3% TCA. Mix.
Label a third test tube as Blank B
Add 1 ml of water and 4 ml of 3% TCA. Mix
Let stand all the three tubes at room temperature for 10 minutes.
Mix and read absorbance of B, S and T at 450 nm or using blue filter.

Calculation·

$$\text{CSF protein in mgs/100 ml} = \frac{T - B}{S - B} \times 50$$

5.2 2.2 Method II: Sulfosalycylic acid method.

Principle.

Sulfosalycylic acid precipitates proteins to produce turbidity The extent of turbidity is proportional to the protein content.

Reagent.

1. Sulfosalycylic acid, 3% (w/v).
   Dissolve 3 g of sulfosalycylic acid in water and make upto 100 ml. Or 3 g of sulfosalycylic acid is dissolved in 7% sodium sulfate solution, and made upto 100 ml with the sodium sulfate solution.
2. 'Stock protein standard solution—5 mgs/ml:
   Dissolve 595.25 mg of bovine albumin and 100 mgs of sodium azide in water and make upto 100 ml. Store at 4^0C in refrigerator.

Procedure:

Label a test tube as Blank (B).
Add 4.5 ml water and 2.5 ml of sulfosalycylic acid. Mix.

Label a second tube as Test T

Add 0.5 ml of serum, 2.5 ml of sulfosalycylic acid and 4.0 ml of water Mix

Label a third tube as standard S.

Add 0 5 ml of working standard, 2.5 ml of sulfosalycylic acid and 4 ml of water Mix.

Let stand the three tubes for 5 minutes at room temperature

Set the colorimeter to zero with Blank.

Read T and S at 420 nm or using blue film

Calculation

$$\text{mgs Total proteins per 100 ml CSF} = \frac{T}{S} \times 50$$

Note·

1 If the CSF protein content is very high better estimate by Biuret method exactly as serum total protein determination.

2. In place of albumin standard serum of known protein content can be used with proper dilution as to get 5 mg/ml protein. Actually serum standard is better than bovine albumin standard.

Interpretation:

Normal CSF has a protein content of 15 to 45 mg per 100 ml. It is elevated in meningeal inflammations, brain tumours and sub-arachnoid hemorrhage.

5.2.3. Determination of CSF Chloride

Method: Schales and Schales

Sample: CSF.

Principle:

CSF is titrated against mercuric nitrate at the end point of which a permanent pink color is obtained.

Reagents·

1. Mercuric Nitrate
   Take 20 ml of double distilled water in a beaker. Add 3 ml concentrated nitric acid. Add 3.2 g of mercuric nitrate to the solution in beaker. Dissolve Transfer the solution in beaker to one litre flask and add water upto 1 litre mark. Mix. The reagents keep indefinitely.

2. Nitric acid approx , IN:
   Dilute 6 ml of concentrated HNO_3 to 100 ml of 95% ethyl alcohol. Stored in a brown bottle in refrigerator, keeps indefinitely.

3. Diphenyl carbazone, 0.1%
   Dissolve 100 mgs of diphenyl carbazone in 100 ml of 95% ethyl alcohol. Stored in brown bottle in refrigerator keeps indefinitely.

4 Ethyl Ether.

5 Standard chloride solution 100 mEq/1
   Dry some sodium chloride (AR grade) crystals at 110°C for 2-3 hours in a hot-air-oven. Allow it to cool in a desiccator. Weigh 5.845 g of dry sodium chloride and dissolve in double distilled water and dilute to one litre.

Procedure.

Test:

Take a test tube and label it Test (T).
Pipet 2 ml of water, 0.2 ml of CSF.
Add 1 drop of 1 N HNO_3 and 3 drops of dephenyl carbazone.
Add 1 ml of ethyl ether. Mix

Titrate using mercuric nitrate taken in a 2 ml pipet (calibrated to 0.01 ml) to the appearance of permanent violet color. Note the titration value (mls) mercuric nitrate.

Standard·

Into a test tube labeled Standard (S) pipet 2 ml water, 0.2 ml standard solution.

Add 1 drop 1 N HNO_3 and 3 drops of diphenyl ether. Mix. Titrate using the same pipet taking mercuric nitrate in it. Note the titration value (mls of mercuric nitrate)

Calculation:

$$\text{meq/L of chloride in CSF} = \frac{\text{Titration of test}}{\text{Titration of std.}} \times 100$$

Note:

1. Use double distilled water throughout the work.
2. No appreciable difference is observed without the use of ethyl water.
3. Sodium chloride for standard is dried at 110°C in air-oven for 2-3 hours. Then it is allowed to cool in desiccator

Interpretation:

Normal CSF chloride level is between 120 and 130 mEq/L. Decrease in chloride in CSF is found in meningitis. In tuberculous meningitis CSF chloride is lowered whereas in viral meningitis it is normal

5.2.4. Pandy's Test with CSF

Principle.

The globulin is precipitated by phenol which is evidenced by the opalescence formation.

Sample: Cerebrospinal fluid.

Reagent:

Pandy's solution.
Dissolve 10 grams of phenol in 150 mm distilled water.

Procedure:

Pipet 2 ml Pandy's solution in a test tube. Add two drops of CSF Mix Observe.

Result:

The test is reported as "no opalescence", "Marked opalescence' and "turbid" depending on the extent of opalescence formation.

Interpretation:

Normal CSF shows no opalescence. This test gives a rough idea of the increase in globulin.

Increases are seen in general paralysis of the insane, tabis dorsalis and disseminated sclerosis.

PART VI

LABORATORY MAINTENANCE AND IMPROVEMENT

6.1 Quality Control

Test results of Biochemistry laboratory are used for diagnosis and treatment of disease. Erroneous results may mislead in diagnosis resulting in unnecessary medical treatment and prolonged hospitalisation. Unnecessary hospitalisation puts extra-financial burden on the patient and brings a bad name to the medical man, the institution as well. Therefore the quality of such data must be controlled The laboratory result must be fully reliable and to attain this every effort must be made for constant checking and search for error.

A high degree of quality control is achieved through cleanliness of glasswares, carefully designed requisition slips and report forms, identification and labeling of samples and recording the data and of course, high degree of precision and accuracy.

The International Federation of Clinical Chemistry's Expert Panel defines quality control as "The study of those errors which are the responsibility of the laboratory and the procedures used to recognise and minimise them. This study includes all errors arising within the laboratory between the receipt of the specimen and the despatch of the report; on some occasions, the responsibility of the laboratory may extend to the collection of the specimen from the patient, and the provision of a suitable container".

A careful and continuous check is necessary at:

1. Cleanliness of glasswares,
2. Quality of glasswares—A grade pipettes,
3. Instrument—maintenance,
4. Chemicals—Use of AR or GR grade,
5 Method of determination,
6. Clinical and other errors,
7. Quality of work—precision and accuracy.

The quality of work done in a laboratory is assessed or evaluated

by having a close look at the precision and accuracy, when a single sample is estimated several times and when these values are close together it refers to good precision. Secondly when these values are close together to the standard value of the same sample it refers to good accuracy.

Quality Control Material:

Usually pooled normal serum forms the material for quality control. Pooled lyophilised serum can be got from standard companies. it can also be prepared in the laboratory as follows:

1. Pool the left over serum after the analysis is complete Collect 1-2 liters. Exclude hymolysed jaundice and lipemic (turbid) serum.
2. Filter the pooled serum through glass-wool taken in a funnel.
3. Mix the serum thoroughly
4. Adjust the pH to 7.4. This is done by adding concentrated sulfuric acid carefully with contunuous, vigorous mixing and checking the pH with a pH meter.
5. Distribute 10 ml portions of this into several plastic vials preferably otherwise glass vials. Store in deep freezer. This last for three months.
6. Each day take out one vial for use.

Method:

1. Remove one control serum vial from the deep freezer Thaw it and allow to come to room temperature.
2. Perform the test treating it the same way as sample serum.
3. Determine the value.
4. Pilot the value on the quality control chart.

Conclusion:

1. The value on a particular day falls within ±2 SD (Standard Deviation) indicates all the reagents and standards are okay.
2. In case the value is above or below 2 SD, same reagent or

standard is deteriorated. Check, identify and replace the reagent or standard with a fresh one and again determine the value which falls within ±2 SD.

3. Sometime the control serum itself might be deteriorated Replace with a fresh pool of serum
4. Quality control chart indicate deterioration of reagent or standard or the quality of work

Quality Control Chart:

Levey-Jennings chart is almost universally followed in all the laboratories.

Levey-Jennings chart is prepared for each determination in a laboratory.

Before preparing the chart, a knowledge of standard deviation is a necessity.

Standard deviation, S.D.:

Standard deviation indicate the average error or average deviation. A small deviation is tolerable whereas a wide deviation shows loss of precision or error in reagent which has to be rectified.

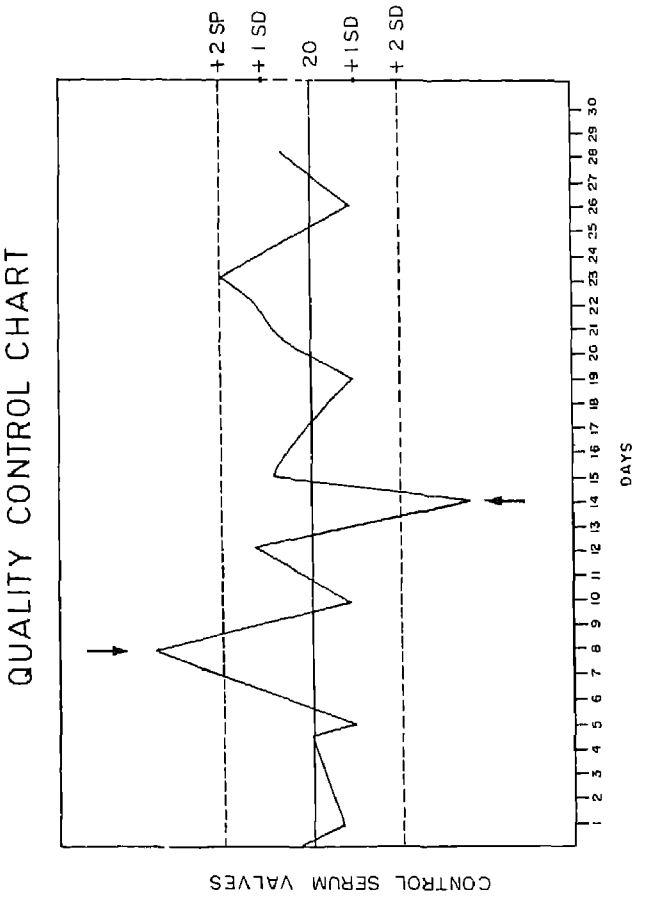
It is calculated as follows:

1. The arithmatic mean ($\bar{X}$) of the value is calculated.
2. Find the difference between the individual value (x) and the mean ($\bar{x}$) i.e. ($\bar{x} - x$). If x is greater than $\bar{x}$ find the difference and drop the sign.
3. Square each of these deviations i.e. $(\bar{x} - x)^2$
4. Add these squares of deviations i.e. $\Sigma (\bar{x} - x)^2$
5. Devide the $\Sigma (x - x)^2$ by the number of values minus one, i.e if there are a number of values then it is

$$\frac{\Sigma (x - x)^2}{n - 1}$$

6. Then the square root of this is S.D., i.e.

$$\frac{\Sigma (\bar{X} - X)^2}{n - 1} = \text{S.D.}$$



The tolerance limit i.e. whether ±2 SD, ±2.5 SD or ±3 SD is left to the laboratory. The chart is prepared on a graph sheet. The graph is drawn by plotting control serum values on y-axis and the days on x-axis.

The following is the composite quality control chart. The mean value for this is 20 and control limits are ±2 SD i.e. 16 to 24. (Fig. VII-20).

On 8th day and on 14th day the value is out of tolerance limits and hence problem exist. A search is made of reagent or standard and rectified.

Maintenance of good quality control:

The following points are essentially considered:

1. Complete methodology is made available at each working table.
2. A technician thoroughly trained is posted for each method.
3. A close and continuous observation is held on storage specifications, expiration dates and details of preparation of reagents.
4. New batch of reagents are introduced after running through control serum and found satisfactory. This is done before the exhaustion of the present reagent.
5. Primary standards are used whenever feasible (Eg. glucose, urea, cholesterol, uric acid, creatinine etc.)
6. Instruments like pH meter, photometer and waterbath are checked frequently for correct performance
7. Each batch of sample determinations should include control serum and standard.
8. All glasswares are thoroughly cleaned.
9. Pipets and volumetric flasks used must be "A" grade quality.
10. Laboratory data are recorded separately for each test in separate books.
11. Experienced staff should be involved in the laboratory activities at all times.
12. The technicians should do the best job and also communicate observations and suggestions to their supervisors for appropriate action.
13. After careful scrutiny only the report forms are signed and despatched.
14. Complete honesty by the entire laboratory staff is very important since questionable results are potentially more harmful to the patient than no results at all.

Improvement:

Every effort is made continuously for improvement of quality of existing work. Improvement aspect is often forgotten because of

being completely absorbed in maintaining the existing degree of quality control. This is achieved by better methods, instruments, training and quality control programmes which should be the concern of the head of the institution.

6.2 Automation and Kits

Automation

Automation means mechanisation of conventional manual technics. In 1957 automation was introduced in clinical laboratory for quantitation of some of the constituents in biological fluids. With the addition of new parameters from time to time to diagnose diseases precisely and the urgency for reports increased the workload so tremendously that it is felt impossible to cope up with time. This situation prompted to resort to automation of manual technics. Thus automation makes possible to do a large number of tests in a great number of samples in comparatively a short period of time. The instrument used in clinical laboratory is called autoanalyzer

A number of manufacturers produce a variety of autoanalyzers. The operating details for each instrument are best found in manuals supplied by the manufacturers. In this chapter is dealt the different types of automated systems and some of thieir advantages and disadvantages.

Types of Autoanalyzers

In any of the autoanalyzers, sample, reagents and standards at intervals are fed and the final values are obtained through a recorder, printer or computer.

There are basically two types of automated system, such as continuous flow analysis and discreate analysis. In the continuous flow system, samples follow each other in sequence through a channel. In this system each sample is separated from the following one with air.

In discreate analysis system each sample occupies a separate container and the containers are tested in parallel or in sequence.

Advantages

Provided the autoanalyzer is basically sound the following advantages are realised.

1. Large amounts of samples may be processed with mininal time.
2. Two or more methods may be performed simultaneously.
3. Precision is far superior to that of manual performance.
4. Calculations may not be required.

Disadvantages

1. Very expensive to purchase and maintain. Regular attention as well as regular visits from trained service personnel is essential.
2. For small numbers of sample autoanalyzer is impractical. Hence manual methods have to be backed up.
3. Sample may be turbid or may be coloured which may interfere with one or the other estimations in which the instrument fails to recognise.
4. Because the instrument is fully automated the technician may fail to notice potential problems in it.
5. There are limitations in the type of methodology which can be used. Sometimes a compromise is made in the chemistry which results in less accurate values than with manual technics.
6. At any time technicians should be ready for estimations with manual techniques.

Kits

A set of ready-made reagents and method of estimation form a kit. Commercialisation of technical skill resulted in the release of kits into the market. Some kits are well designed and reliable while others are not and should be avoided. "The purchase of a kit means that the laboratory buys not only whatever the expertise the manufacturer may possess in regard to the test, but also whatever errors, inaccuracies and general lack of quality that may exist in the kit".

A better kit should provide.

1. Detailed directions.
2 The principle involved in the estimation
3 A description of calculations.
4. Calibration curve.

5. Normal value obtained with the kit.
6. Details of quality control.
7. Expiration dates and storage conditions of reagents.
8 A performance evaluation from a reputed laboratory.

As far as possible it is suggested to prepare the reagents in the laboratory not only for reasons of low costs but to maintain good quality of work.

In cases of tests requested infrequently or difficult to set up and maintain, tend to be more costly and inconvenient, they are the ones in which kits are used

6.3 Laboratory Management

The head of the laboratory should be conversent with many of the acreditation standards and governmental regulations One should not just take up the job of managing a clinical laboratory attracted by the monetary benefits but also one should realise the vast responsibility as those laboratory reports have a direct impact on the health care of patients.

The clinical laboratory management requires expertise in medical scientific and technical areas The person should have resources in the form of personnel, equipments, supplies; facilities and skills in organisation, management and communication.

A clinical laboratory should stand on firm goals and objectives. In order to achieve these objectives, the clinical laboratory should have adequate facilities, equipment and supplies and adequate number of personnel.

The objectives of a clinical laboratory is to assist the medical professionals with reliable reports of tests—the reliable test reports of clinical laboratory is a vital component of excellent health care delivery system. Such being the case every effort is made at maintenance and improvement of quality of work.

Laboratory management can be well understood on the following heads

1. **Space and design:**
   A laboratory should be spacious with good ventilation and lighting system A well designed worktables avoids confusions.

2. **Equipment and facilities:**
   Basic instrument and equipment requirements should be made available As far as possible some of the vital instruments should be in duplicates.

   Water and power supply should be adequate. For purposes of speedy communication telephone should be provided Safety

measures like providing fire-extinguisher, hoods or smoke chambers, first-aid kit and chart are essential.

3. **Procurement:**
One should decide what supplies they need, when they need them and in what quantities. Much time can be wasted unless a systematic procurement system is developed For this the addresses of various manufacturers must be procured

Equipment, chemicals and other supply-market is so competitive that only through product investigations can the best product be selected within the available funds This is best done in consultation with several other clinical laboratories.

4. **Storage and maintenance:**
Every personnel in a laboratory should be thoroughly familiar with storage specifications irrespective of chemicals, reagent or instruments

A constant check is maintained on expiration dates Chemicals and reagents after the expiration dates should be disposed of without delay to prevent impending hazards

a. Chemicals and reagents:—Storage specifications mentioned for the chemicals by the manufacturer and the expiration of dates are strictly followed.

   i) Chemical balance should be checked for accuracy and sensitivity periodically This is done with a sophisticated balance with high sensitivity, if available, or by comparing with a set of standard weights.

   ii) pH meter should be checked monthly for correct performance. This is done with standard buffers prepared afresh. The electrode is washed with organic solvent like alcohol occasionally.

   iii) Colorimeters and spectrophotometers are checked periodically for correct performance at different wavelengths using different solutions of known O.D. The phototube is replaced when it shows loss of sensitivity which is evidenced by low O.D for the known standard solutions

   iv) Water-bath and oven temperatures shouled be checked often.

v) Distilled water should be checked for ions like chloride, sulfate, phosphate, calcium etc.

vi) Voltage fluctuations in the mains are better overcome with the use of stabilizers.

vii) Perfect cleanliness and discipline should be maintained in the laboratory

5. Personnel:

a. Qualified persons should be recruited.

b. A good rapport should be maintained between the person-in-charge and the working staff.

c Basic needs and comforts should be provided

d. The personnel is given the job-description.

e. Duty allotment schedule among the staff should be carefully prepared.

f. Opportunities should be provided for betterment of skills and position.

PART VII SELECTED REFERENCES

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4. **Instrumentation**
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5. **Interpretation of Results**
   (1) Varley, H., Practical Clinical Biochemistry.
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   (3) Widman, F.F., Clinical Interpretation of Laboratory tests